BIOCHEMICAL GENETICS OF HYDROGEN METABOLISM IN *Escherichia coli*: *hydB*, *hydF* and *fhlA* GENES

By

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BIOCHEMICAL GENETICS OF H_2 METABOLISM IN *Escherichia coli*: *hydB*, *hydF* and *fhlA* GENES

By

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Mutant strains of *Escherichia coli* which lost hydrogenase activity and hydrogen dependent functions were found to carry mutations in two unlinked genetic loci, namely, *hydA* and *hydB*. These mutations were mapped at 59 min of *E. coli* chromosome. In this study, the *hydB* genetic locus was studied in detail. The *hydB* locus contains four linked genes, namely, *hydB*, *hydF*, *fhlA* and *fdv*. Gene-products of *hydB* and *hydF* are essential for hydrogenase and hydrogen dependent activities, while *fhlA* gene is needed for formate dehydrogenase-2 (FDH-2) and formate hydrogenlyase (FHL) activities. A cloned recombinant plasmid, pSE-128, carries all four genes. The *hydB* gene is found to be contained within a DNA segment of 800 basepairs and codes for a 32,000 dalton protein produced only under anaerobic conditions.

The *hydF* gene is about 1,000 basepairs in length. The gene-product of *hydF* gene has not been identified. The polarity of *hydF* gene has been proposed.

The gene-product of *fhlA* is needed for both formate dehydrogenase-2 (FDH-2) and FHL activites. This gene codes for a 78,000 dalton protein which is produced under aerobic and anaerobic conditions. The *fdv* gene codes for a 82,000 dalton protein whose function is not known. The *fdv* gene-product is also produced both aerobically and anaerobically. The direction in which *fhlA* and *fdv* genes are transcribed has been determined. The physiological roles of all these genes in H_2 metabolism of *E. coli* are areas of future research.

INTRODUCTION

The ability to metabolize hydrogen gas is found in many prokaryotes (3), and in some eukaryotes such as green algae (71) and protozoa (42). The prokaryotic microorganisms include several economically important bacteria, such as methanogens, nitrogen-fixers, as well as fermentative bacteria (3). Hydrogen is a metabolic by-product of many anaerobic bacteria during fermentation of sugars (3). Photosynthetic production of hydrogen by cyanobacteria and green algae, using sun light and water, has been proposed as a potential source of fuel and chemical feedstock (35). Hydrogen also serves as a source of reducing power and energy for both aerobic and anaerobic bacteria, under conditions of energy limitation (3). Archaebacteria use hydrogen as a reductant for the production of methane (5). Reutilization of H_2 by nitrogen-fixing organisms is also known to enhance the energy efficiency of nitrogen fixation process (18).

Inspite of the important role hydrogenase plays in bacterial metabolism and its potential use in biotechnology for fuel production and nitrogen fixation, very little is known regarding the biochemical genetics of hydrogen

metabolism even in a well studied bacterium such as Escherichia coli. Until 1985, there was no defined selection procedure available for isolation of mutant strains of E. coli defective in H_2 metabolism. This limited the genetic studies on this important metabolic pathway.

The availability of a positive selection procedure for isolation of mutant strains, defective in hydrogen metabolism, made it possible to isolate a large number of mutant strains altered in H_2 metabolism in E. coli (40). Analysis of these mutant strains helped identify several genes involved in H_2 metabolism in E. coli (54). These genes are found grouped in four loci in the E. coli chromosome. This study presents the biochemical and genetic characterizations of the genes in one such locus.

REVIEW OF LITERATURE

In 1931, Stephenson and Stickland proposed the name hydrogenase (EC 1.12) for the enzyme which catalyzes the production and consumption of H_2 (59). Since that time, hydrogenase activity was demonstrated in several bacteria (3), green algae (71), protozoa (42) and in higher plants, such as barley (62). The protein had been purified from several bacteria and characterized (3). The enzyme hydrogenase catalyzes a reversible reaction as outlined below:

$H_2 + e^-$ carrier (oxidized) == $2H^+ + e^-$ carrier (reduced)

Hydrogenase plays a vital role in the anaerobic metabolism of microorganisms. In fermentative bacteria, in the absence of exogenously added electron acceptors, H_2 evolution by hydrogenase helps to oxidize the reduced electron carriers produced during fermentation.

In E. coli, a facultative anaerobe, pyruvate metabolism, yields formate and acetyl CoA. The formate is further metabolized to produce H_2 and CO_2 by the enzyme complex formate hydrogenlyase which includes formate dehydrogenase-2, hydrogenase and intermediate electron carriers (35). In Clostridium pasteurianum, a strict

anaerobe, pyruvate, generated from glucose catabolism, is broken down to yield acetyl CoA, CO_2 and H_2 via pyruvate:ferredoxin oxidoreductase and hydrogenase (3, 63). In this organism, excess NADH generated at the glyceraldehyde-3-phosphate dehydrogenase level is also oxidized to produce H_2 by NADH:ferredoxin oxidoreductase and hydrogenase.

Hydrogenase also catalyzes the oxidation of H_2 when H_2 is the only source of reducing power and energy for the cell. The electrons derived from the oxidation of H_2 by hydrogenase are shuttled through electron carriers to reduce an ultimate electron acceptor such as fumarate in *E. coli* (9) or SO_4^{2-}, as in the case of *Desulfovibrio* (46). In *Paracoccus denitrificans*, nitrate can serve as the terminal electron acceptor for the reducing power generated from the oxidation of H_2, while nitrate represses hydrogenase activity in *E. coli* (58). Methanogens utilize H_2 as a reductant to reduce CO_2 to methane (5). This activity of hydrogenase, generally called H_2 uptake (HUP) activity, generates energy for the growth of the organism.

Photosynthetic bacteria also utilize H_2 as a source of reducing power to support CO_2 fixation (32). Some of the non-sulfur purple bacteria are known to evolve H_2 in the dark, fermentatively, as in *E. coli* (23). Both photosynthetic bacteria and aerobic hydrogen-oxidizing bacteria, such as *Alcaligenes* spp. can grow autotrophically,

with H_2 as sole electron donor. Several members of the genus Alcaligenes produce a cytoplasmic hydrogenase which can reduce NAD^+ directly. The NADH can be coupled to the reduction of O_2 to H_2O or can serve as a reductant for CO_2 fixation (57). Alcaligenes also produces a membrane-bound hydrogenase, whose presumptive function in the cell is O_2-dependent oxidation of H_2 coupled to ATP production (19, 57). These few examples, indeed, demonstrate the importance of hydrogenase(s) in the physiology and metabolism of a diverse group of microorganisms.

The enzyme hydrogenase is usually associated with electron carrier proteins and exists in the cell as part of a multienzyme complex catalyzing unidirectional reactions. However, hydrogenase, once purified, does catalyze the oxidoreduction reaction reversibly, in the presence of suitable, artificial, electron donors or acceptors (3, 34).

Hydrogenase from Clostridium pasteurianum was the first to be purified to homogeneity (11). Since these initial studies, hydrogenases from several other bacteria have been purified (3). Based on the molecular weight and the number of subunits, purified hydrogenases from several sources can be grouped into three categories; i) a single polypeptide chain with a molecular mass ranging from 50,000 to 66,000 daltons; ii) a protein comprising two non-identical subunits with a native molecular mass ranging from 89,000 to 101,000 daltons; 62,000 to 67,000 daltons for a large subunit and

26,000 to 34,000 daltons for the small subunit; iii) a third group of hydrogenase found in Paracoccus denitrificans and Alcaligenes eutrophus is a tetramer with two large subunits (63,000 to 67,000 daltons) and two small subunits (31,000 to 33,000 daltons).

Hydrogenase is a protein containing non-heme iron and acid labile sulfur (3). Hydrogenases also contain nickel, although exceptions to the presence of nickel had been reported (3, 26). The presence of iron and nickel in the holoenzyme dictates the need for processing of hydrogenase apoprotein into holoenzyme. Hydrogenases, like other non-heme iron and sulfur proteins, are usually sensitive to oxygen and are irreversibly inactivated (3). Although the hydrogenase protein from several organisms is capable of tolerating O_2, the enzyme from Clostridium pasteurianum and Desulfovibrio gigas is extremely sensitive (11, 46).

Two hydrogenase activities have been demonstrated in E. coli; H_2 evolution (FHL hydrogenase) and H_2 uptake (HUP hydrogenase). Several investigators observed that crude extracts obtained from E. coli cells, grown under different growth conditions, exhibited multiple hydrogenase bands in non-denaturing polyacrylamide gels stained for hydrogenase activity, suggesting the possibility of iso-enzymes in the cell (1, 69).

More recently, Ballantine and Boxer (6) using immunological procedures, demonstrated two antigenically

distinct hydrogenase isoenzymes in E. coli containing nickel. These investigators also proposed the presence of a third hydrogenase isoenzyme in E. coli (55). Sawer and Boxer (56) purified the hydrogenase isoenzyme-1 to near homogeneity and found that it consisted of 2 polypeptides of molecular mass 64,000 and 35,000 daltons. Ballentine and Boxer (7) purified an active fragment of the second hydrogenase and found that the native molecular mass of the enzyme was 180,000 daltons, comprising 2 each of non-identical subunits with molecular mass of 61,000 and 35,000 daltons. On the other hand, Adams and Hall (2), and Patel (48) purified a hydrogenase, that is presumably the H_2 uptake hydrogenase consisting of a single polypeptide with a molecular mass of 56,000 daltons.

Studies on the genetics of hydrogenases and hydrogen metabolism have only recently received great attention (36, 50, 54, 70). In 1975, Pascal and her co-workers (47), using a dye-overlay method, isolated and described a mutant strain of E. coli which lacked hydrogenase activity and mapped the mutation at 57 min of E. coli chromosome. However, this mutant strain was found to be defective in other enzymes in the metabolic pathway such as formate dehydrogenase-1 and -2. Graham et al. (24) isolated two mutants of E. coli with similar characteristics, using a similar dye-overlay procedure. Karube et al. (30, 31) described a mutant strain of E. coli which also mapped at

this location. Krasna (36) isolated a hydrogenase mutant of E. coli defective in all H_2 dependent functions. A Mud 1(Ap lac) insertion mutation, termed ant (anaerobic electron transport) was mapped at 59 min between mutS and srl genes of E. coli. This strain lacked the ability to produce H_2 (FHL), but had hydrogenase activity (7Ø). Mutations in pyruvate formatelyase (pfl) gene (13) or in a regulatory gene, fnr (fumarate, nitrate reduction) (39), were also found to influence the production of hydrogenase activity by the cell.

Our laboratory developed and employed a unique positive selection procedure to isolate a large number of mutants defective in H_2 metabolism in E. coli and separated them into two major classes (4Ø, 6Ø). Class I mutant strains were found defective in H_2-uptake activity, but they possessed formate hydrogenlyase activity. The mutation in class I mutant strains was mapped at 65 min of the E. coli chromosome. The class II mutants lacked hydrogenase and all the H_2-dependent activities and the mutation was mapped at 59 min. Subsequently, it was demonstrated by cloning and complementation analysis that the class II mutant strains can be further separated into two unlinked groups, hydA and hydB. The hydB gene region was shown to contain 3 genes, namely, hydB, fhlA and fdv (54).

Wu and Mandrand-Berthelot (68) isolated hyd mutant strains and mapped the mutations at 77 min of E. coli

chromosome. Mutation in one of the genes, hydC, was suppressed by exogenously added nickel while a mutation in a nearby gene, hydD, was not. The function of the hydD gene is unknown, at the present time. Waugh and Boxer (67) reported a hyd mutation which abolished hydrogenase activity. This mutation, which mapped in the hydB region, was also restored to wild type character by the addition of nickel to the growth medium. Later it was shown that a hyd mutant strain (AK-23) isolated by Krasna (36) was similar to that of Waugh and Boxer's strain (67) because the mutant phenotype of strain AK-23 was also suppressed by exogenously added nickel. This gene, involved in nickel metabolism, has been termed hydE.

At least, seven hyd genes, whose gene products are essential for hydrogenase activity, have been identified in E. coli. Among them hydC and hydE are involved in nickel transport into the cell. None of the other genes affecting hydrogen metabolism, identified and studied in E. coli, was shown to be the structural gene for hydrogenase, although the structural gene for hydrogenase (H_2-uptake hydrogenase) has been isolated and characterized from other organisms [Rhizobium japonicum (72), Desulfovibrio vulgaris (Hildenborough) (65), D.gigas (12), and D. baculatus (44)].

The H_2 metabolic pathway involves several proteins such as pyruvate formatelyase, formate dehydrogenases, hydrogenases, fumarate reductase, ubiquinone, several

unknown electron carrier proteins and regulatory proteins. Hydrogenases, formate dehydrogenases, fumarate reductase and some of the electron carriers are non-heme Fe and acid labile sulfur proteins. Hydrogenase holoenzyme also contains nickel and formate dehydrogenase is a molybdo-selenoprotein. Metals such as Fe, S, Mo, Se, and Ni have to be transported into the cell and processed to convert the apo-proteins into active holoenzymes. It is conceivable that these genes are involved in the regulation of production and/or processing of apo-hydrogenase to active hydrogenase in the cell. A complete study of the genes involved in the regulation of H_2 metabolism is an important step in understanding this process.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co. Inorganic and organic chemicals were obtained from Fisher Scientific Co. and were analytical grade. Restriction endonucleases and T4 DNA ligase were obtained either from Bethesda Research Laboratories (BRL) or New England BioLabs. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals (BMB). Exonuclease Ba131 was purchased from either BMB or New England BioLabs.

Bacterial strains, bacteriophages and plasmids are listed in Table 1. All bacterial strains are derivatives of E. coli K-12.

Abbreviations

HF medium (H_2-fumarate) is a chemically defined medium used to test the ability of E.coli cells to grow under anaerobic conditions, utilizing H_2 gas as electron donor and fumarate as electron acceptor. HUP (H_2 uptake) indicates the ability of E. coli cells to use HF medium for growth. HUP activity is defined as the utilization of H_2 as an electron

Table 1. Bacterial strains, bactriophages, and plasmids used in this study

Strain	Relevant genotype or phenotype	Reference
Bacteria		
K-1Ø	Hfr PO2A relA1 pit-1Ø tonA22 $T2^r$ λ^+ SpoT	4Ø
Puig 426	thi-1 leu-6 suc-1Ø bioA2(?) galT27 rpsL129 chlC3 λ^-	4Ø
JC 1Ø244	alaS cysC43 srl-3ØØ::Tn1Ø thr-1 leu-6 thi-1 proA2 galK2 ara-14 xyl-5 mtl-1 lacY1 his-4 argE3 rpsL31 tsx-33	4Ø
SE-1ØØØ	JC 1Ø244 $alaS^+$	4Ø
SE-38	Puig 426 hydB1Ø3	54
SE-53	Puig 426 hydA1Ø1	54
SE-65	Puig 426 hydF1Ø5	54
SE-67	Puig 426 hydF1Ø7	54
SE-68	JC 1Ø244 hydB1Ø8 fhlA1Ø1	54
SE-1174	SE-38 $hydB^+$ fhlA1Ø2::Tn1Ø	
MBM 7Ø14	F^- araC(Am) araD (argF-lacU169) trp(Am) mal(Am) rpsL relA thi supF	8
CSR 6Ø3	F^- thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara14 lacY1 galK2 xyl-5 mtl-1 gyrA98(nalA98) rpsL31 tsx33 $\lambda^=$ supE44	53
MC 41ØØ	araD139 Δ(argF-lac)2Ø5 flb853Ø1 relA1 rpsL15Ø deoC1 λ^-	37
Phage		
λ 421	b221 rex::Tn5 CI857 Oam23 Pam8	37

Table 1 continued.

Strain	Relevant genotype or phenotype	Reference
Plasmid vectors		
pBR-322	*tet bla*	1Ø
pUC-19	*bla lacZY*	64
pSE-4	*tet*, *bla*, pSa origin of replication	

donor with fumarate as the electron acceptor. Hup is denoted to represent H_2 uptake phenotype (Hup^+ and Hup^- represent the wild type and mutant phenotypes, respectively) which includes hydrogenase, fumarate reductase, and other electron carriers required for H_2 consumption. The gene symbol hyd is used for hydrogenase, and hup is used to designate the genes whose products are essential for H_2 uptake. Gene symbol fdv represents the gene product which is essential for formate dehydrogenase (FDH) activity that couples formate oxidation to reduction of artificial electron acceptor benzyl viologen (BV). This formate dehydrogenase (FDH-2) is a component of the formate hydrogenlyase (FHL) enzyme complex. The gene symbol fhl is used to designate genes, the products of which are needed for production of FHL activity.

Media and Growth Conditions

Luria broth was prepared as described previously (40). Glucose minimal medium had the following composition: Na_2HPO_4, 6.25 g; KH_2PO_4, 0.75 g; NaCl, 2.0 g; $FeSO_4.7H_2O$, 0.01 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; $NaMoO_4.2H_2O$, 0.01 g; Na_2SeO_3, 0.263 mg in 1 liter of deionized water. The final pH of the medium was 7.5. Glucose, autoclaved separately, was present at a final concentration of 3.0 g/L for aerobic cultures and 15.0 g/L for anaerobic cultures. The HF medium was the same as described by Bernhard and

Gottschalk (9). Solid medium contained 15 g of agar per liter of medium. Ampicillin, kanamycin and tetracycline, when present, were added to the medium, after autoclaving, to a final concentration of 100, 50, and 15 ug/ml, respectively.

For growth under H_2, bacterial cultures were spread on the surface of the HF medium in petri dishes, and the plates were placed in a vacuum desiccator. The gas phase was removed and replaced with H_2, and the plates were incubated at room temperature. Anaerobic conditions were established in the desiccator within 18 to 24 hours, as determined by an anaerobic indicator strip (Gas Pak; BBL Microbiology Systems). Colonies were observed in about 5 to 7 days. All cultures employed for enzyme assays were grown at 37°C, except the strains carrying alaS mutation. The alaS mutant strains were grown at 30°C.

Biochemical Experiments

Protein was determined by Folin phenol method, described previously (40) with bovine serum albumin as the standard. The enzyme activities are expressed as nanomoles of product produced or substrate oxidized per minute per milligram of protein.

Whole Cell Enzyme Assays.

Aerobically grown stationary phase cells were used as inoculum [5% (vol/vol)]. The cells were grown anaerobically in 20 ml of LB with 0.3% glucose in 16 X 150 mm tubes. The tubes were filled to the top to achieve anaerobic conditions and incubated, at 37°C, for 4 h (temperature sensitive mutant strains were grown at 30°C, for 5 h). The cells were collected by centrifugation, at 3,500 X g, for 10 min, at room temperature and washed once with 5.0 ml of wash buffer [phosphate buffer, 0.1 M, (pH 7.0) containing 1 mM reduced glutathione and 100 ug/ml of chloramphenicol, to prevent continued protein synthesis, during the assay]. The cells were suspended in 1.0 ml of wash buffer and maintained in ice, under N_2 atmosphere. Hydrogenase and formate dehydrogenase activities were measured at 550nm as reduced BV formation, using an UV-Vis spectrophotometer (SLM-Aminco-DW2C) by monitoring the reduction of BV.

Hydrogenase. Hydrogenase activity was determined by two different methods. First was an exchange reaction using $^3H_2$ as an analog of H_2. This reaction, $^3H_2 + H^+ == {}^3H^+ + {}^3H\text{-}H$, is independent of electron transport proteins (35) and provides a direct measure of the hydrogenase activity present in the cell. Hydrogenase activity was also determined by the rate of reduction of BV by H_2, in the

presence of hydrogenase, a common procedure used by other investigators (47).

Tritium exchange assay. Whole cells, grown as described above, were washed once with wash buffer and assayed at a cell protein concentration of 50 to 100 ug/ml, in a tube (12 X 75 mm). The final assay volume was 0.2 ml. The tube was sealed with a serum stopper and the gas phase was replaced with helium. Into this tube, 0.7 ml of H_2 gas was injected, after removing 0.7 ml of helium. Tritium gas (25 ul; 11.2 mCi/mmol; New England Nuclear Corp.) was added to a final concentration of 0.55 uCi per tube. After one hour of incubation, at room temperature, the serum stopper was removed, and the tritium gas was vented out in the hood for 10 min, after vigorous mixing of the tube contents. Tritiated water present in a 50-ul sample was determined with a scintillation counter, in Aquasol-2 scintillation fluid. Hydrogenase activity was expressed as nanomoles of tritiated water produced, per hour, per milligram of cell protein.

Hydrogenase-dependent BV reduction. The reaction mixture contained NaK-PO_4 buffer (0.1 M; pH 7.0), BV (50 mM), and the cell suspension in a final volume of 3.0 ml in an anaerobic cuvette. The gas phase was replaced with H_2. The rate of BV reduction was determined at 550 nm.

Hydrogen uptake activities were also determined by monitoring the disappearence of H_2 from the gas phase by using a gas chromotograph, in the presence of either BV or fumarate, as electron acceptor. The assay mixture for these reactions contained, in a final volume of 1.Ø ml, NaK-PO_4 buffer (Ø.1 M; pH 7.Ø), BV or fumarate (5Ø mM), and cell suspension at a final concentration of 15Ø to 2ØØ ug of cell protein, in a 1Ø ml wheaton vial (4Ø). The gas phase was replaced with N_2, and H_2 was added to a final concentration of 1Ø%. The amount of H_2 in the gas phase was determined at different time intervals, with a Gas Chromatograph (Varian model 92Ø). The activity was expressed as nanomoles of H_2 consumed, per minute, per milligram of cell protein.

FDH-2. The assay mixture contained, in a final volume of 3.Ø ml, NaK-PO_4 buffer (Ø.33 M, pH 7.Ø), BV (6.5 mM), sodium formate (Ø.1 M) and cell suspension. The rate of reduction of BV was monitored.

FHL. The reaction mixture for FHL assay contained NaK-PO_4 buffer (Ø.33 M, pH 6.5), sodium formate (Ø.1 M) and cell suspension (25Ø to 3ØØ ug of total cell protein), in a final volume of 1.Ø ml. The rate of H_2 evolution with time was monitored by using a gas chromatograph.

The enzyme activities were expressed as nanomoles of BV reduced, per min, per mg of cell protein, using a molar

extinction coefficient of 7.78 X 10^3 cm^{-1}. Formate Hydrogenlyase activity and H_2 uptake activity (HUP) were expressed as nanomoles of H_2 produced or consumed, respectively, per minute per milligram cell protein.

Enzyme Assays with Cell Extracts.

Preparation of cells and extracts for enzyme assays. Cells used for enzyme assays were grown in 1.0 liter of LB medium, under anaerobic conditions, by filling the culture vessel to the top. Aerobically grown stationary phase LB-cultures were used as inoculum [5% (vol/vol)]. For preparation of extracts, cells from 4 h old cultures were harvested by centrifugation at 8000 X g, for 10 min, at 4°C. The cells were washed once with wash buffer and centrifuged again at 12,000 X g (4°C) for 10 min. The cells were resuspended in 1.0 ml of wash buffer and passed through a French pressure cell at 20,000 lb/in^2. The broken cell suspension was centrifuged at 20,000 X g, for 20 min, at 4°C and the supernatant was collected. This crude extract was maintained in ice, under an N_2 atmosphere.

FDH-2. The assay mixture for FDH-2 contained NaK-PO_4 buffer (0.33 M, pH 7.0), BV (6.5 mM), sodium formate (40 mM) and the cell extract. The final volume was adjusted to 3.0 ml with deionized water (21). The reaction was carried out

in an anaerobic cuvette, at room temperature, in an N_2 atmosphere and the rate of BV reduction was monitored.

FHL. The reaction mixture for FHL assay contained NaK-PO_4 buffer (0.33 M, pH 6.5), sodium formate (40 mM), and cell extract (25) in a final volume of 1.0 ml. The reaction was carried out at room temperature, with N_2 in the gas phase. The rate of production of H_2 was monitored with a gas chromatograph (Varian, model 920) equipped with a molecular sieve 5A column.

Genetic Experiments

Cloning the hyd Gene. Total chromosomal DNA was isolated from a prototrophic strain of E.coli K-12 strain K-10 (52). Plasmid pBR322 (10) was used as a vector in the cloning experiments. Plasmid DNA was isolated as described by Davis et al. (16). A gene bank containing E.coli chromosomal DNA fragments was constructed by the general procedures described by Ditta et al. (17). Chromosomal DNA was partially digested with the restriction endonuclease Sau3A at a concentration of 0.1 unit of the enzyme per ug of DNA, for 15 min, at 37°C (54). The Sau3A digestion was stopped by incubating the reaction mixture in a 65°C water bath for 10 min. The digested DNA sample was layered on a 36 ml, 10 to 40% linear sucrose gradient. The sucrose gradient was made with 9.0 ml each of 10, 20, 30, and 40% (wt/vol) sucrose

solution [containing Tris-HCl (20 mM, pH 8.0), EDTA (10 mM) and NaCl (50 mM)] layered gently on top of each other starting from 40% sucrose at the bottom of the centrifuge tube. The sample was centrifuged in a SW 27 rotor, at 23,000 rpm for 18 h, at 25°C. The gradient was fractionated by collecting 1.0 ml samples, using an ISCO-density gradient fractionator (Model 183). The DNA samples containing sizes larger than 10 kb were pooled together. Agarose gel electrophoresis was used to determine the size of the DNA fragments, using HindIII-digested phage lambda DNA as standard. The vector, plasmid pBR322, was digested with the restriction endonuclease BamHl and the 5' end of the linear DNA was dephosphorylated with calf intestinal alkaline phosphatase (0.01 unit/pmol of 5'end of DNA for 1h at 37°C). This dephosphorylated and linearized vector DNA was ligated with Sau3A-digested chromosomal DNA of size above 10 kb, using T4 DNA ligase. A hydrogenase-defective, recA strain of E.coli, strain SE-61, was transformed (10) with the ligation mixture, and the ampicillin-resistant colonies were selected in LB medium supplemented with ampicillin. To isolate the plasmids containing the hydrogenase genes, the Ap^R colonies were transferred to HF medium by replica plating methods. The Hup^+, Ap^R colonies were selected and maintained. The plasmid present in these clones was extracted and tested for hyd and other relevant characters. Total plasmid DNA from the pool of Ap^R transformants was also isolated separately

and maintained as an E. coli gene-bank. Starting with this E. coli "gene-bank" DNA, plasmids capable of complementing the hyd/hup mutants belonging to other classes and not represented by strain SE-61 (e.g., strain SE-53) were also isolated by the procedures described above.

Mapping the Genes in Recombinant Plasmids.

In order to identify the number of genes present in the chromosomal DNA insert, physical maps of the plasmids, were constructed based on restriction endonuclease digestion pattern, using previously published procedures (54). Restriction endonuclease digestion conditions were either as described by Davis et al. (16) or as recommended by the manufacturer of the enzyme.

Construction of Physical Map of Plasmid DNA by Restriction Endonucleases.

Plasmid DNA can be identified by he presence of a specific gene and also by the presence of unique restriction endonuclease cleavage sites (33). Based on the location of these unique sites in the linear DNA, different plasmid DNA molecules can be differentiated. In this section, a strategy used for constructing one such map is presented. Using this general strategy, all other plasmids were characterized.

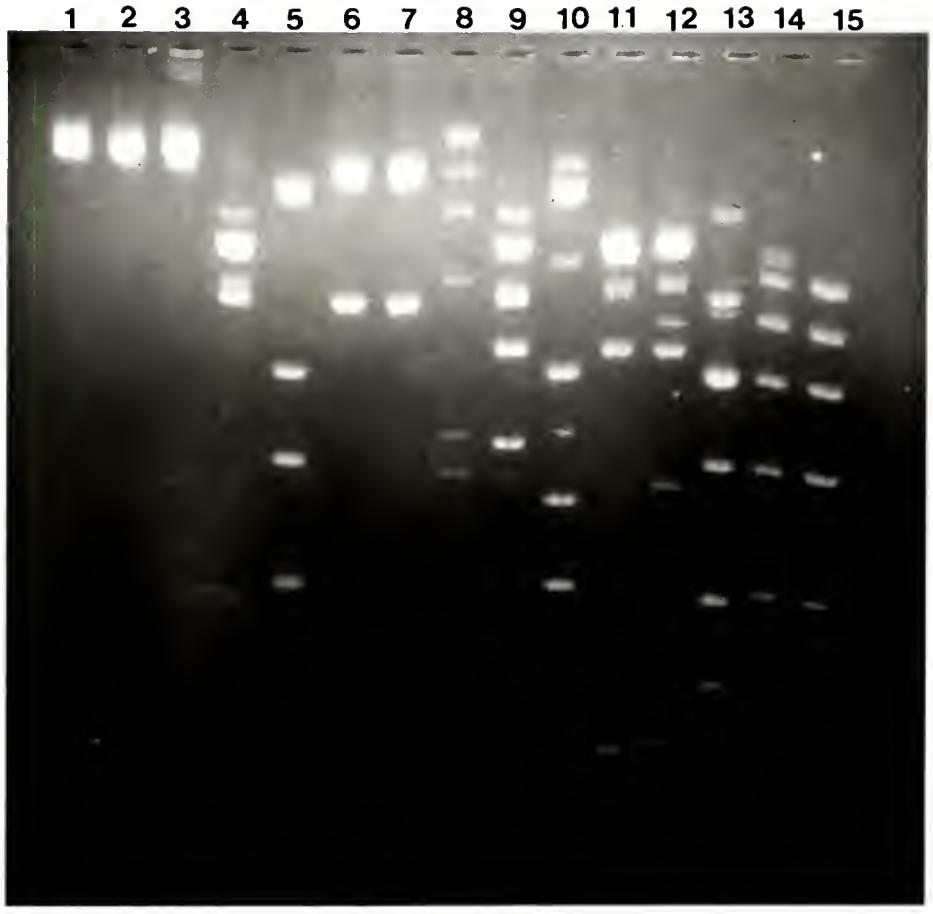

Fig. 1. Agarose gel electrophoresis of plasmid pSE-22 digested with various restriction endonucleases (see text for details). The gel was cast with Ø.8% agarose and contained lug/ml of ethidium bromide. Lanes: 1, BamHI; 2, EcoRI; 3, HindIII; 4, PstI; 5, SalI, 6, BamHI-HindIII; 7, BamHI-EcoRI; 8, phage lambda DNA digested with HindIII; 9, PstI-BamHI; 1Ø, SalI-BamHI; 11, PstI-EcoRI; 12, PstI-HindIII; 13, PstI-SalI; 14, SalI-HindIII and 15, SalI-EcoRI.

Plasmid DNA was digested with various restriction endonucleases, independently and in combination with other restriction endonucleases to generate DNA fragments. These fragments were then subjected to electrophoresis in Ø.8% agarose gel. DNA fragments migrate in this gel based largely on the size of the fragments. Plasmid DNA (plasmid pSE-22) was digested with various restriction endonucleases as described by Davis et al. (16). Procedures used for preparation of agarose gel, and for electrophoresis were described previously (43). In this example, an agarose gel, showing the electrophoretic characteristics of DNA fragments from restriction endonucleases-digested plasmid pSE-22, is presented in Fig. 1.

Bacteriophage lambda DNA, digested by enzyme HindIII, was used as a standard for molecular weight determination. The enzyme HindIII digested the phage DNA at 7 locations and released 8 fragments: 23.76, 9.46, 6.67, 4.26, 2.25, 1.96, Ø.59, and Ø.1Ø kilo base-pairs (kb). However, in the gel only 6 fragments are visible. The last two fragments were not detected because these fragments were smaller in size for detection, under these experimental conditions. Using the molecular weights of HindIII-digested phage lambda DNA fragments and the relative migration (Table 2), a standard curve was developed. Using this standard curve, the sizes of restriction enzyme

Table 2. Enzyme HindIII digested phage lambda DNA (lane #8, Fig. 1)

Distance Migrated (centi-meters)	Fragment Size (kilo base-pairs)
Ø.75	23.76
1.ØØ	9.46
1.35	6.67
1.95	4.26
3.3Ø	2.25
3.6Ø	1.96

Table 3. Restriction fragments of plasmid pSE-22 generated by restriction endonucleases (Fig. 1)

Lane#	Enzyme(s) used	Distance migrated (cm)	Calculated Fragment Size (kb)
1	BamHI	Ø.95	11.Ø6
2	EcoRI	Ø.95	11.Ø6
3	HindIII	Ø.95	11.Ø6
4	PstI	1.75	4.85 (X2)
		2.15	3.86
5	SalI	1.3Ø	7.Ø5
		2.8Ø	2.8Ø
		3.5Ø	2.ØØ
		4.55	1.21
6	BamHI-HindIII	1.2Ø	7.73
		2.2Ø	3.74
7	BamHI-EcoRI	1.15	8.18
		2.2Ø	3.74
8	HindIII (phage lambda)	see Table 2.	
9	PstI-BamHI	1.7Ø	5.Ø8
		2.1Ø	3.94
		2.6Ø	3.Ø3
		3.38	2.12
1Ø	SalI-BamHI	1.3Ø	7.Ø5
		2.8Ø	2.8Ø
		3.85	1.7Ø
		4.6Ø	1.18
		7.55	Ø.29
11	PstI-EcoRI	1.8Ø	4.7Ø (X2)
		2.6Ø	3.1Ø
		6.ØØ	Ø.61
12	PstI-HindIII	1.7Ø	5.1Ø (X2)
		2.6Ø	3.11
		5.9Ø	Ø.64

Table 3 continued.

Lane#	Enzyme(s) used	Distance migrated (cm)	Calculated Fragment Size (kb)
13	PstI-SalI	2.1Ø	3.94
		2.8Ø	2.8Ø (X2)
		3.55	1.97
		4.7Ø	Ø.97
		5.4Ø	Ø.8Ø
		7.18	Ø.35
14	SalI-HindIII	1.95	4.32
		2.3Ø	3.56
		2.8Ø	2.8Ø
		3.6Ø	1.89
		4.65	1.15
15	SalI-EcoRI	2.Ø3	4.1Ø
		2.4Ø	3.41
		2.9Ø	2.68
		3.65	1.89
		4.7Ø	1.12

fragments from plasmid pSE-22 (Fig.1) were deduced and are listed in the Table 3.

In mapping plasmid DNA, the sizes of fragments generated by a single restriction enzyme digest are compared with sizes of fragments generated by two enzymes-digest. In the two enzymes-digest, one of the enzymes is the same as used in the single enzyme digest. In this above example, sizes of DNA fragments generated by enzyme SalI (lane #5) was compared with sizes of fragments obtained after double-enzyme-digests, such as SalI-BamHI (lane #10); SalI-EcoRI (lane #15); SalI-HindIII (lane #14) and SalI-PstI (lane #13). From these results, the sites for other enzymes (BamHI, EcoRI, HindIII, and PstI) in plasmid pSE-22 were assigned in relation to SalI sites in the plasmid DNA. Similarly, by comparing the sizes of fragments generated by other enzymes, the sites of these restriction enzymes could be obtained (Fig. 1, Table 3). By super-imposing these data, a restriction map of plasmid pSE-22 was deduced (Fig. 3).

Subcloning. In all subcloning experiments, any one of the vectors, such as, plasmid pBR-322 (10), pUC-19 (64), or pSE-4 (this study) was used depending on the suitability of a particular vector for a given experiment. DNA for ligation was obtained by 95% ethanol precipitation after multiple extractions with phenol:chloroform and ether. To the DNA sample, half the volume of 7.5 M ammonium acetate was added,

mixed and to this 95% ethanol was added at 2 times the aqueous volume. This sample was maintained at -20°C for at least 2 h before precipitating the DNA by centrifugation for 30 min, in an Eppendorf centrifuge, at 12,000 X g. After draining the supernatant, 1.0 ml of 70% ethanol was added to the tube to wash off the remaining salt. The DNA pellet obtained after centrifugation was air dried. An appropriate volume of sterile deionized water was added to dissolve the pelleted DNA.

Subcloning was achieved primarily by two different ways. The chromosomal DNA insert was partially digested by single restriction endonuclease which has more than one recognition site in the DNA insert and none in the vector part of the plasmid, followed by self-ligation. After self-ligation, the resulting plasmid would be deleted for different segments of chromosomal DNA present in the plasmid (54).

Alternatively, the recombinant plasmid DNA was digested with one or more restriction endonucleases and various insert fragments were recloned. The DNA sample was subjected to gel electrophoresis in a low-melting agarose (Sigma Chemical Co.) gel. After electrophoresis, the desired band of DNA was removed with the gel. The gel was heated to 65°C, for 10 min. To this sample, 2X volume of sterile deionized water was added, mixed gently and extracted with equal amounts of phenol saturated with Tris-HCl (10mM, pH 8.0) and EDTA (1mM) buffer, for 20 min. The sample was centrifuged,

at 12,000 X g, in an Eppendorf centrifuge, for 15 min, at 4°C. The top aqueous layer was extracted 2-3 times with phenol:chloroform (1:1 vol/vol). The phenol was removed from the DNA sample by extracting few times with ether. Ammonium acetate (7.5 M) was added at a concentration of one half of the volume. The DNA was precipitated with ethanol as above. This DNA was ligated to appropriately digested vector plasmid.

Physical mapping of genes by deletion was accomplished by two different means. In the first procedure the restriction endonuclease Sau3A was used to partially digest the DNA fragments. The digested DNA was ligated with appropriately digested vector DNA. Recombinant plasmids obtained from E. coli cells transformed with this DNA were characterized. In the second technique, exonuclease Bal31 was used to digest the insert DNA progressively from one end, after linearizing the plasmid with an appropriate restriction endonuclease (43). At various time intervals, small samples of reaction mixture were removed, added to 200 ul of stop buffer [EDTA (0.1 M, pH 8.0), SDS (0.1%)] and maintained for at least 5 min at 50°C to terminate the reaction. Alternatively, the Bal31 digested DNA sample, obtained as before, was subjected to gel electrophoresis in low-melting agarose. The desired size fragments were removed from the gel and purified as described before. The purified DNA fragments were 'blunt-end' ligated (43), transformed

into appropriate mutant strains and the isolated plasmid DNA was characterized.

Transposon Tn5 Mutagenesis of Various Genes in a Recombinant Plasmid (pSE-128).

The recombinant plasmid pSE-128 was transformed into E. coli K-12 strain MBM 7014 (supF). Phage lambda 421 carrying the transposon Tn5 was used to introduce Tn5 into this strain by transduction (45). For these experiments, the strain MBM 7014 was grown in 10.0 ml of LB supplemented with maltose (0.3%), to a cell density of 2 X 10^8 CFU/ml. The cells were centrifuged and resuspended in 1.0 ml of 10 mM $MgSO_4$. A 0.2 ml sample of this cell suspension was infected with phage lambda 421 at a multiplicity of infection (MOI) of 1.0, for 30 min, at room temperature. The cells were spun down and washed once with 2.0 ml of LB. These cells were suspended in 20.0 ml of LB with glucose (0.4%) and sodium citrate (10 mM), in a 250 ml Erlenmeyer flask. The culture was shaken at 200 rpm, for 30 min, at 37°C. After this time, 30 ug/ml of kanamycin and 100 ug/ml of ampicillin were added and the culture was shifted to 30°C and left shaking at 200 rpm, over night. Plasmid DNA was extracted from this culture and used to transform hyd mutant strains. Transformants were selected on LB-agar containing kanamycin (50 ug/ml) and ampicillin (100 ug/ml). The Kan^R and Ap^R transformants were tested for Hup and Fhl characteristics. Individual Hup^- and Fhl^- colonies were selected. Plasmid present in these clones

were extracted and transformed into the same strain to confirm the plasmid genotype.

Small Scale Plasmid Preparation. For routine small scale plasmid preparation, two different procedures were used. The first procedure is a modification of a method described by Maniatis et al. (43). An overnight culture (1.5 ml) was transferred to an Eppendorf tube and spun for 2 min. The pellet was suspended in Ø.1 ml of ice-cold Sucrose-EDTA-Tris buffer (SET) with lysozyme. SET buffer contained Tris (25 mM, pH 8.Ø), EDTA (1Ø mM), and sucrose (5Ø mM). To the required volume of this buffer, EDTA (1.Ø M, pH 8.Ø) and lysozyme were added to a final concentration of Ø.1 M and 4 mg/ml respectively. The sample was mixed and incubated at room temperature, for 5 min. After this period, Ø.2 ml of freshly prepared lysis buffer (Ø.2 N NaOH, 1% SDS) was added and the tube contents were mixed gently for a few seconds. After 5 min of incubation, in ice, Ø.15 ml of potassium acetate buffer was added, mixed gently and incubated for an additional 5 min, in ice. The potassium acetate buffer contained 6Ø ml of 5.Ø M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of water to the final concentrations of 3.Ø M potassium and 5.Ø M acetate ions. The pH of this buffer was adjusted to 4.8. The sample was centrifuged in an Eppendorf centrifuge, at 12,ØØØ X g, for 5 min, at 4^{o}C. To the supernatant, Ø.Ø5 ml of Ribonuclease A

(1 mg/ml, heat treated at 100°C for 2 min to inactivate any contaminating deoxyribonuclease) was added and incubated for 10 min at room temperature. This sample was extracted twice with phenol:chloroform (1:1 vol/vol). The plasmid DNA was precipitated with ethanol as before, after the removal of phenol with ether.

In the second procedure, cells carrying plasmid DNA were grown in 10 ml of LB over night (10). The cells were spun down at 4°C and suspended in 0.3 ml of Tris-HCl (0.05 M, pH 8.0) buffer containing 25% glycerol. To this cell suspension, 0.04 ml of 1.0 M EDTA and 1 mg of lysozyme were added. The sample was incubated in ice for 10 min. About 1 mg/ml of pronase, 0.02 ml of 20% SDS, and 0.05 ml of Ribonuclease A (1 mg/ml) were added to the spheroplast preparation and incubated in ice for 10 min. The sample was transferred to a small centrifuge tube for extraction with an equal volume of cold phenol saturated in TE buffer (Tris 10 mM, pH 8.0; EDTA 1 mM) for 10 min. The top aqueous layer was obtained after centrifugation and extracted twice with phenol:chloroform, ether and the DNA was precipitated with ethanol as described before.

Identification of Proteins Produced from Plasmid-Encoded Genes (Maxi-Cell Experiment).

The general procedure used for determining the proteins produced by recombinant plasmids is as described by Sancar et al. (53). E. coli maxi-cell K-12 strain CSR-603 was

transformed with the plasmids. The transformation was achieved by a modification of the method described by Bolivar et al. (1Ø). The cells were grown in 1Ø.Ø ml of LB at 3Ø^{o}C until the optical density corresponded to 5 X 1Ø^{7} CFU/ml. The culture was shifted to 37^{o}C until the cell density reached 1 to 2 X 1Ø^{8} CFU/ml. The cells were spun down at room temperature and washed once with 5.Ø ml of Ø.1 M NaCl. The cells were suspended in 5.Ø ml of Ø.1 M $CaCl_2$ and incubated at room temperature for 2Ø min. The cells were spun down again and resuspended in 1.Ø ml of Ø.1 M $CaCl_2$ and left in ice for 12 h. In a pre-chilled tube, Ø.2 ml of cells was taken and plasmid DNA was added at a final concentration of 1ØØ ng per tube. The tube contents were incubated in ice for 3Ø min and shifted to 42^{o}C for 2 min. To this sample, 1.Ø ml of LB was added and incubated at 37^{o}C for 1 h. The cells were centrifuged and resuspended in Ø.1 ml of LB and plated on selective medium containing appropriate antibiotics.

The transformants (strain CSR 6Ø3 with plasmids) were grown in K-9 medium (53) supplemented with MOPS buffer (5Ø mM; pH 7.Ø). For aerobic expression of the genes in the plasmid, 2.5 ml of aerobically grown culture (2 X 1Ø^{8} CFU/ml) was transferred to a sterile petri-dish and exposed to 1.4 uW/cm^{2}.sec of ultra violet irradiation (254 nm) for 5Ø sec. The intensity of UV radiation was measured using an ultra-violet meter (DM-245N, Spectroline). The

irradiated culture was transferred to a 125 ml screw cap flask. Freshly prepared cycloserine was added to the culture at a final concentration of 200 ug/ml and shaken at 200 rpm for 16 h.

For anaerobic expression of the genes in a plasmid, 10 ml of cells were grown in the same medium as above, but under N_2, in a 70.0 ml of serum-stoppered vial. The cells were irradiated with UV (1.4 uW/cm^2.sec) under a constant stream of N_2 flowing over the culture. The irradiated culture was transferred to a 25 ml Erlenmeyer flask containing 20 ml of the same medium, pre-flushed with N_2. This medium also contained 200 ug/ml of freshly prepared cycloserine. The flask was shaken (100 rpm) at 37^oC for 16 h. In the absence of shaking, cells settled down at the bottom of the flask resulting in survival of cycloserine-sensitive cells. After 16 h, the cells from both cultures were centrifuged at room temperature and washed twice with Hershey salts medium (53). The anaerobic culture was handled with minimium exposure to O_2. These cells were suspended in 1.0 ml of Hershey medium and incubated for 1 h, at 37^oC, to starve them for sulfur, in order to label the cells with ^{35}S-methionine (1,086 Ci/mmol, NEN). For labelling with ^{35}S-methionine, $MgSO_4$ was replaced with $MgCl_2$ and 50 ug/ml of required amino acids were added. The starved cells were labelled with 5 uCi of ^{35}S-methionine per tube for 1 h, at 37^oC. The labelled cells were spun down and

washed twice with 1.Ø ml of Ø.1 M NaCl and resuspended in Ø.1 ml of 1X solubilization buffer (38). The 2X solubilization buffer contained in 5 ml, 2.Ø75 ml of Tris (Ø.1 M, pH 7.Ø), 1.5 ml of 1Ø% SDS, 1.Ø ml of glycerol, Ø.25 ml of beta-mercaptoethanol, and Ø.ØØ5% bromocresol purple. The lysed cells were incubated in a boiling water bath for 2 min and spun down in an Eppendorf centrifuge at 12,ØØØ X g, for 15 min. A 3 ul sample of the supernatant was removed and counted in a scintillation counter after adding 2.5 ml of scintillation fluid, ScintiVerse E (Fisher scientific Co.).

A sample of the extract with a radioactivity of 5Ø,ØØØ to 1ØØ,ØØØ cpm was subjected to polyacylamide gel electrophoresis (PAGE), in 12% acrylamide gels (38). After electrophoresis, the gels were treated with either Enhance or Enlighting (New England Nuclear) as outlined by NEN. In some experiments, the gels were also treated with PPO using the procedure developed by Jen and Thach (28). One side of the gel was covered with Whatman 3mm chromatography paper and the other side with all-purpose polyvinylchloride wrap. These gels were vacuum dried at 8Ø^{o}C, for 1 h and 3Ø min, using Eprotec senior (Haake Buckler) gel drier. The dried gels were exposed to Kodak X-ray film, XR-5, in a Kodak X-Omatic cassette with intensifying screeens on both sides, at -8Ø^{o}C, for few hours to 1 day as needed. The X-ray film was developed using Kodak GBX-developer and fixer. Protein molecular weight standards (Mark-VII, Sigma) were also run

in the same polyacrylamide gel, in a separate lane. The lane with the molecular weight standards was cut out after the run, processed and stained with coomassie blue as described previously (38). The molecular mass of the radioactive proteins were determined after comparing the Rf (relative mobility is defined as the ratio between the distance of protein migration and distance of tracking dye migration) of the sample to the standards.

RESULTS

Cloning hyd Genes.

The class II mutant strains of E. coli, described previously, lacked hydrogenase and were defective in all hydrogen dependent activities (40). The mutation in these strains was mapped between 58 and 59 min (srl and cysC) of E. coli chromosome. It was demonstrated (40), by bacteriophage P1-mediated transduction, that these mutant strains could be divided into two groups, namely, hydA and hydB (Fig. 2). In order to further establish the fact that these two operons are separate and independent, mutant strains from representative groups were transformed with the plasmid DNA from the "gene-bank" constructed and described in the Methods section. The mutant strains, SE-53 (hydA101) and strain SE-68 (hydB108, fhlA101) were plated on LB medium containing ampicillin, after transformation with the plasmid DNA from the "gene bank." Ampicillin resistant colonies were transferred to HF medium by replica-plating techniques and incubated for 5 days at room temperature, under H_2 atmosphere. Individual HUP^+ colonies were selected and maintained. The presence of hyd/hup genes in the plasmid DNA was confirmed by transformation of the same recipient strain

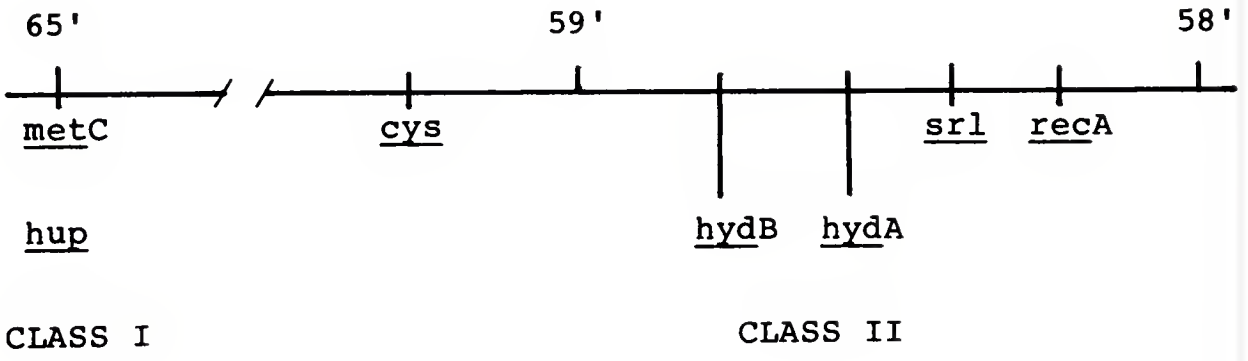

Fig. 2. Genetic map location of the genes essential for H_2 metabolism in E. coli.

by isolated plasmid DNA. These transformants were characterized biochemically for enzyme activities, namely, by the tritium exchange, by hydrogen dependent reduction of BV and by fumarate-dependent H_2-uptake. The results of these experiments are presented in Table 4. The hydrogenase production by the strain SE-53 was complemented by the plasmid pSE-290 and not by plasmid pSE-111. The plasmid pSE-111 was able to complement the strain SE-68 for hydrogenase-dependent activities, while the plasmid pSE-290 failed to do so. The presence of the recombinant plasmids in the wild-type parent, Puig 426, did not influence either the hydrogenase or H_2-uptake activity.

The physical map of the two plasmids (pSE-290 and pSE-111), based on the restriction endonuclease digestion, is presented in Fig. 3. This figure also contains the restriction map of two other smaller plasmids, pSE-201 and pSE-22, capable of complementing hydA and hydB mutant strains, respectively. Plasmid pSE-22 carries a 9 kilo base-pairs (kb) chromosomal DNA insert which is also present within the 14 kb insert in the plasmid pSE-111. The plasmid pSE-201 carries a 5.5 kb insert which overlaps part (4.8 kb) of the 12.3 kb insert in plasmid pSE-290. Based on this restriction analysis no overlap between the two groups of plasmids (plasmids pSE-111 and pSE-201 or pSE-290) was detected (Fig. 3). These results, obtained with the cloned

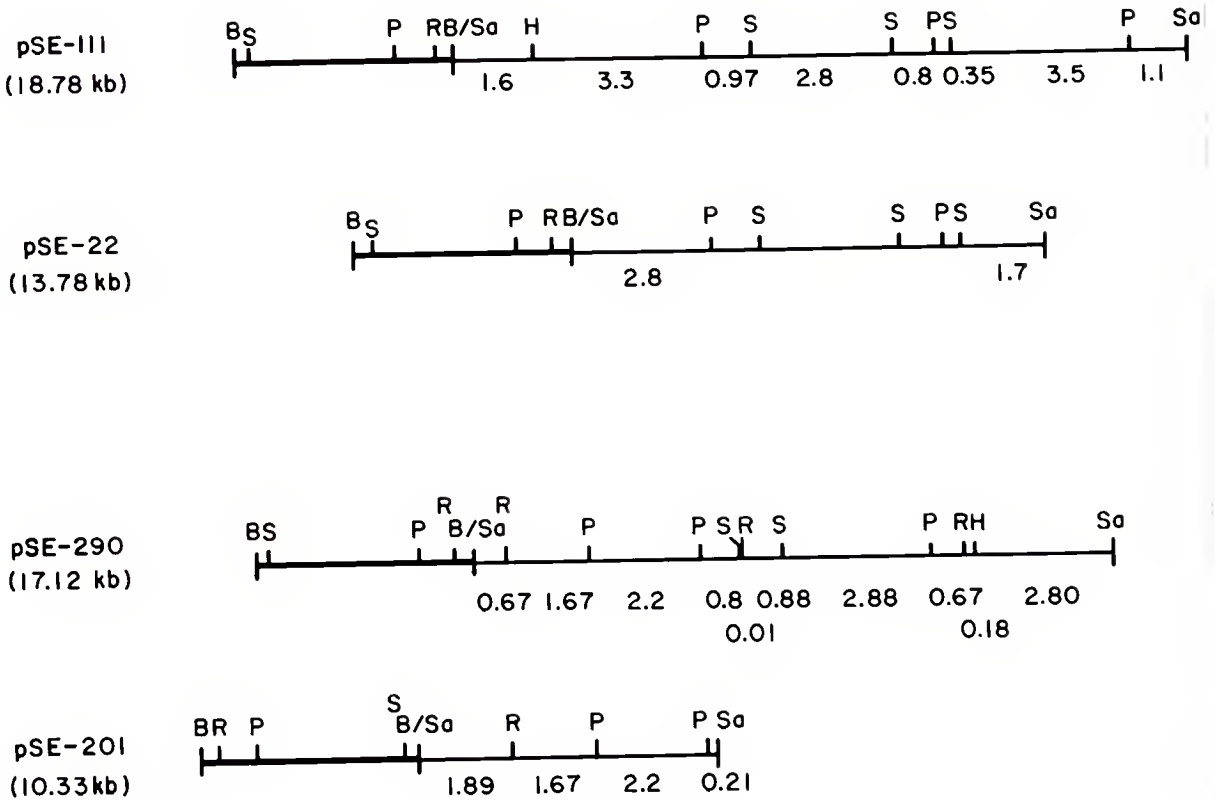

Fig. 3. Restriction map of E. coli recombinant plasmids containing the two different hydrogenase operons. Plasmid pBR-322 (heavy lines) is the vector. Only the relevant restriction sites are marked for the vector. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI; B/Sa, BamHI-Sau3A junction of the vector and chromosomal DNA insert. The numbers indicate the size of the DNA fragment between two restriction sites in kb.

Table 4. Complementation analysis of *hyd* mutants by recombinant plasmids

Strain	plasmid	Hydrogenase activity		
		$^3H_2$ exchange	H_2 to BV	H_2 uptake (Fum.)
Puig 426	-	1,110	427	144
	pSE-111	1,110	340	175
	pSE-290	1,198	446	158
SE-53	-	5	U.D.	U.D.
	pSE-111	9	N.D.	N.D
	pSE-290	1,135	742	183
SE-68	-	18	U.D.	U.D.
	pSE-111	1,030	703	202
	pSE-290	15	N.D.	N.D.

$^3H_2$ exchange-nanomoles of tritium exchanged per h per mg protein.
H_2 to BV-nanomoles of BV reduced per min per mg protein.
H_2 uptake-nanomoles of H_2 consumed per min per mg protein.
U.D, undetectable
N.D, not done

DNA fragments, are in complete agreement with the genetic evidence on the presence of two operons for hydrogenase activity in the cell.

To further understand the organization of genes in the hydB region, a fine structure analysis of the plasmid pSE-22 was carried out and presented below.

Subcloning of the plasmid pSE-22. Plasmid pSE-22 was digested with restriction endonucleases SalI or PstI. The results of this experiment are presented in Fig. 4. Complete digestion of the plasmid pSE-22 with enzyme SalI produced four fragments of sizes 1.15, 2.Ø, 2.8,and 7.8 kb. The largest fragment contained the vector and a 3.45 kb fragment of chromosomal DNA. Circularizing this DNA fragment with T4-DNA ligase yielded plasmid pSE-127. The restriction enzyme SAlI digestion of plasmid pSE-22, followed by ligation produced plasmid pSE-128. Plasmid pSE-128 contained the 2.8 and 7.8 kb fragments only. The 2.8 kb SalI fragment present in plasmid pSE-128 was found to be reversed in plasmid pSE-129. The orientation of this 2.8 kb fragment in the plasmid was established after determining the location of the KpnI and ClaI sites in the fragment (Fig. 4). This 2.8 kb SalI fragment was also removed from plasmid pSE-22 and ligated into the SalI site of the vector plasmid pBR-322 (1Ø). The resulting plasmids, pSE-125 and pSE-126 (Fig. 4) differed in the orientation of the fragment and this was

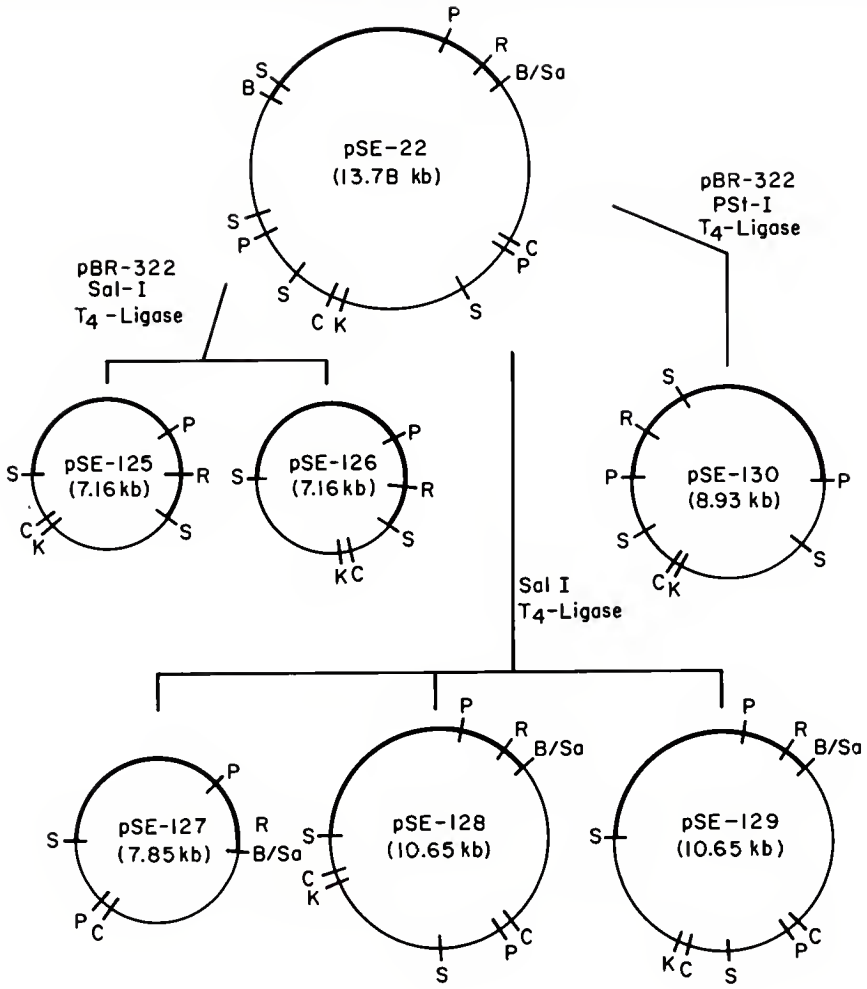

Fig.4. Isolation of plasmid derivatives from plasmid pSE-22 (see Methods section for details)

Table 5. Hydrogenase activities in hyd mutant strain SE-38 in the presence of different subclones of plasmid pSE-22

Strain	Plasmid	Hydrogenase[a]
Puig 426	-	1,110
SE-38	-	<1
	pSE-125	1,574
	pSE-126	551
	pSE-127	1
	pSE-128	447
	pSE-129	605
	pSE-130	719

a, nanomoles of tritium exchanged per h per mg cell protein.

demonstrated by the location of ClaI and KpnI sites in the insert DNA with respect to the EcoRI and HindIII sites in the vector. In constructing plasmid pSE-130, a 4.53 kb PstI fragment which contained the 2.8 kb SalI fragment internally, was ligated into the PstI site of the vector plasmid pBR-322 (Fig. 4).

Characterization of hydB Gene

Strain SE-38 (hydB) was used in the study of hydB region of DNA (Fig. 2) as a representative of several mutant strains whose mutations were mapped in this region (54). The plasmid derivatives obtained from plasmid pSE-22 were used to transform strain SE-38 and the transformants were selected on LB medium containing ampicillin or tetracycline (for selecting plasmid pSE-130). The Ap^R or Tet^R transformants were assayed for hydrogenase activity (Table 5), using cell-free extracts of these transformants. Plasmids pSE-125, pSE-126, pSE-128, pSE-129 and pSE-130 complemented the hydrogenase defect in the strain SE-38, while plasmid pSE-127 failed to do so. These results suggest that the 2.8 kb SalI fragment present in the plasmid pSE-125 (Fig. 4) contains the hydB gene. The same insert present in plasmid pSE-126, although present in reverse orientation, (Fig. 4) was able to complement the defect in strain SE-38,

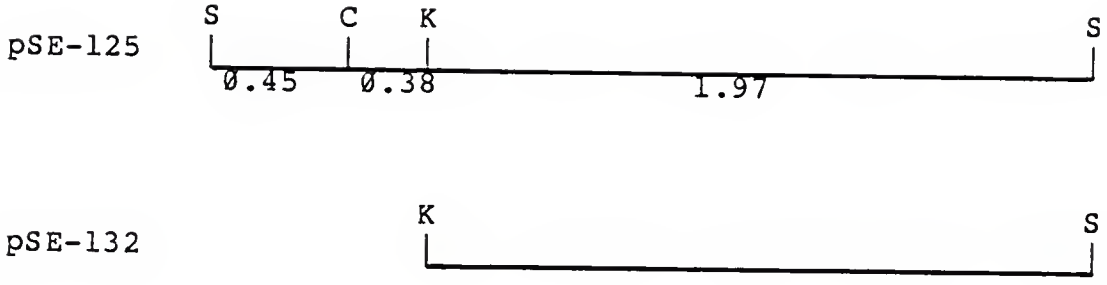

Fig. 5. The chromosomal DNA insert present in plasmid pSE-132. The KpnI-SalI fragment from pSE-125 was cloned into plasmid vector pUC-19 (64). The vector in the plasmid pSE-125 was plasmid pBR-322 (10). The vectors are not shown. The restriction sites are C, ClaI; K, KpnI; and S, SalI. The numbers below the line represent the sizes of DNA fragments in kilo basepairs.

Table 6. Biochemical properties of the hydB mutant strain SE-38 containing plasmids pSE-125 and pSE-132

Strain	Plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-38	-	6.03	3.02	1.58	26.53
	pSE-125	1,521.23	1,289.42	131.65	160.73
	pSE-132	1,545.92	1,312.51	165.75	169.11

a, nanomoles of tritium produced per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.

suggesting that the control region for hydB gene was present within the 2.8 kb SalI fragment.

In order to "fine-structure" map the hydB gene various methods, such as sub cloning and deletion analysis, were sought. From the plasmid pSE-125, a 1.97 kb KpnI-SalI fragment (Fig. 4) was removed and ligated into appropriately digested vector plasmid pUC-19 (64). The resulting plasmid pSE-132 (Fig. 5) was able to complement the hydB mutation present in several strains and the biochemical characteristics of strain SE-38 carrrying plasmid pSE-132 are presented in Table 6. Complementation of the hyd defect in strain SE-38 also restored both FHL and FDH-2 activities. The enzyme activities presented in Table 6 were obtained using whole cells.

In order to identify the location of the hydB gene, within this 1.97 kb insert DNA fragment present in the plasmid pSE-132 (Fig. 5), different regions of this insert DNA were deleted. This was accomplished by partial digestion of the 1.97 kb KpnI-SalI DNA by the enzyme Sau3A and ligation of the resulting fragments to either KpnI and BamHl or SalI and BamHl-digested vector plasmid pUC-19. This digestion-ligation experiment yielded several plasmids carrying deletions from either side of the insert DNA. Some of the plasmids constructed in this experiment are presented in Fig. 6. Plasmid pSE-147 lost the smallest part (Ø.15 kb)

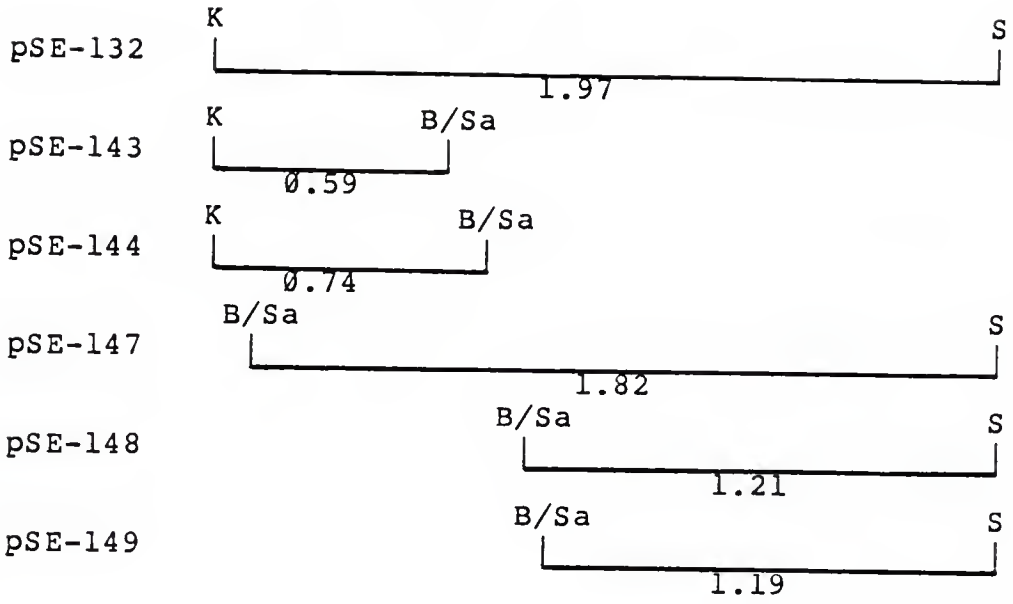

Fig. 6. Chromosomal DNA inserts present in different deletion clones obtained after the enzyme Sau3A partial digestion of the KpnI-SalI fragment of plasmid pSE-132. The vector plasmid used is plasmid pUC-19 (64) and is not shown. Plasmid pUC-19 was digested with KpnI-BamHI for construction of the plasmids pSE-143 and pSE-144 and SalI-BamHI for constructing the plasmids pSE-147, pSE-148 and pSE-149 (see Methods section for details). The restriction enzyme sites are B/Sa, BamHI-Sau3A junction; K, KpnI; and S, SalI. The numbers below the lines represent the sizes of DNA fragments in kilo basepairs.

Table 7. Complementation of mutant strain SE-38 by recombinant plasmids constructed by "Sau3A-deletion"

Strain[a]	plasmid[b]	HYD[c]	HUP[b]	FHL	FDH-2
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-38	-	66.03	3.02	1.58	26.53
	pSE-143	25.37	U.D	0.60	17.32
	pSE-144	74.68	U.D	1.07	15.98
	pSE-147	1,411.21	1,389.96	152.88	50.19
	pSE-148	37.02	U.D	0.85	10.37
	pSE-149	32.68	U.D	0.34	U.D

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomloes of H_2 produced per min per mg protein.
U.D, undetectable

of the insert DNA. Plasmids pSE-143 and pSE-144 had retained Ø.59 kb and Ø.74 kb of the insert from the KpnI end, respectively. The size of the inserts in the plasmids pSE-148 and pSE-149 were 1.21 and 1.19 kb, respectively, extending from the SalI end. Biochemical properties of the strain SE-38 carrying these plasmids are presented in Table 7. The only plasmid that complemented the hyd defect in strain SE-38 was plasmid pSE-147. All other plasmids failed to complement the hydB mutation in strain SE-38. These results suggest that a fragment larger than 1.21 kb is necessary for this complementation.

Since the restriction endonuclease Sau3A has a unique four nucleotide substrate specificity for digestion of the duplex DNA, this method can only generate fixed unique length deletions (33). On the other hand, enzyme Bal31, an exonuclease, can progressively remove the nucleotides in the linear DNA and this reaction can be controlled by the ratio between the concentrations of DNA and the enzyme and also by varying the time of incubation (43). Since partial digestion of the DNA by enzyme Sau3A failed to yield the smallest fragment of DNA needed to complement the hydB mutation, the exonuclease Bal31 was used to generate deletions. However, the transformants obtained from Bal31 deletion experiments, starting with plasmid pSE-132 (vector plasmid pUC-19), grew

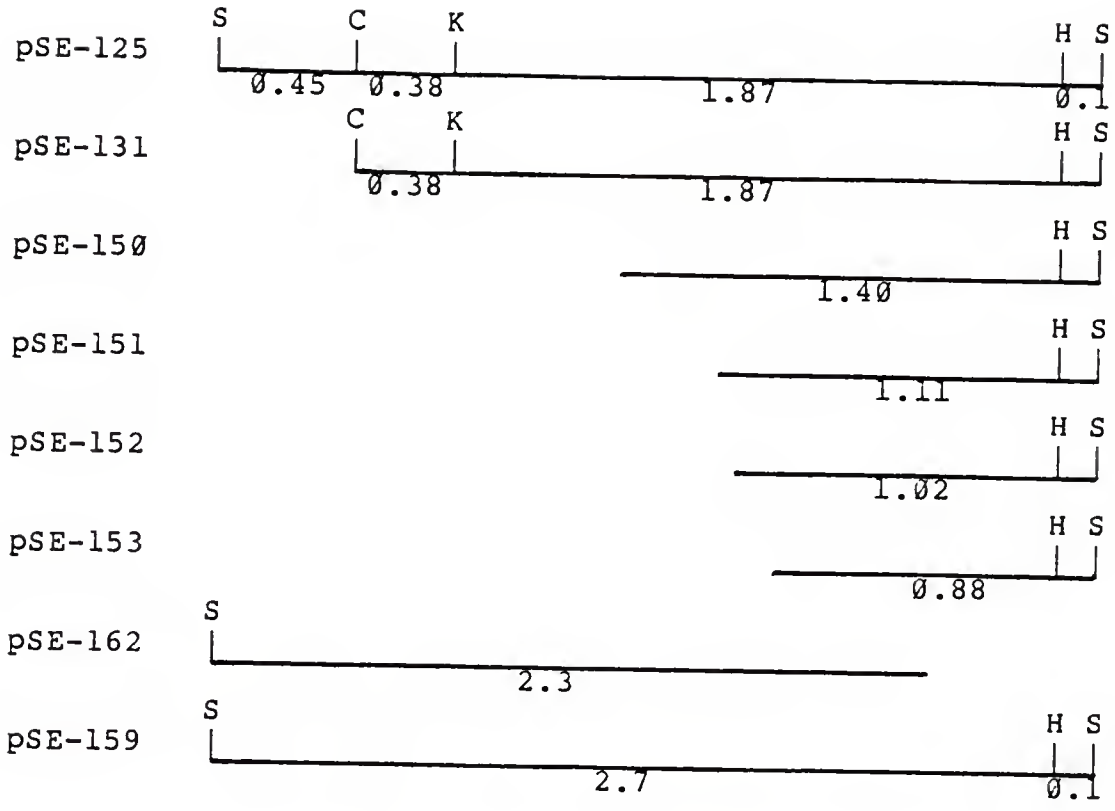

Fig. 7. Chromosomal DNA inserts present in different deletion plasmids obtained after exonuclease Bal31 digestion of plasmid pSE-131, linearized at the ClaI site. Plasmid pSE-162 was obtained by exonuclease Bal31 digestion of plasmid pSE-159, linearized at HpaI site. The vector plasmid present in plasmids pSE-131, pSE-150, pSE-151, pSE-152, and pSE-153 is plasmid pBR-322. The vector plasmid present in plasmids pSE-159 and pSE-162 is plasmid pSE-4. The part of vector plasmids are not shown. The restriction sites are C, ClaI; H, HpaI; K, KpnI; and S,SalI. The numbers below the lines represent the sizes of DNA fragments in kilo basepairs.

Table 8. Complementation of mutant strain SE-38 by recombinant plasmids constructed by "Bal31-deletion"

Strain	plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-38	-	66.03	3.02	1.58	26.53
	pSE-150	2,035.08	1,103.15	83.76	46.41
	pSE-151	1,557.73	818.15	153.02	49.12
	pSE-152	25.55	U.D	0.95	22.97
	pSE-153	27.49	U.D	0.65	15.49
	pSE-162	1,764.70	1,458.05	154.31	149.59

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.
U.D, undetectable

poorly and the plasmids obtained from these transformants had showed various DNA rearrangements. To circumvent these problems, plasmid pSE-131 (Fig. 7) was constructed by ligating the 2.35 kb ClaI-SalI fragment from plasmid pSE-125 (Fig. 5) into the vector plasmid pBR-322. Plasmid pSE-131 was used as the starting parent plasmid in this experiment.

The plasmid pSE-131 was digested with the enzyme ClaI. This linearized DNA was incubated with Bal31 for varying lengths of time. The digested DNA was ligated, transformed into strain SE-38 and Ap^R transformants were selected. In four selected plasmids, pSE-150, pSE-151, pSE-152 and pSE-153, the 2.35 kb DNA was found to be reduced to 1.50, 1.21, 1.12 and 0.98 kb, respectively (Fig. 7). Only plasmids pSE-150 and pSE-151 restored the hydrogenase activity in strain SE-38 (Table 8). Plasmid pSE-152 was smaller (about 0.1 kb) than the plasmid pSE-151 but failed to complement the mutation in strain SE-38. Although plasmid pSE-148 (Fig. 6) carried a similar size DNA fragment (1.21 kb) as that of plasmid pSE-151, it failed to complement the hydB mutation (Table 7). These results suggest that the plasmid pSE-148 lacks the control region (promoter) for hydB gene expression, whereas in the plasmid pSE-151 the control region is present. Although plasmids pSE-147, pSE-150 and pSE-151 complemented the hydB defect in strain SE-38 for hydrogenase, they failed to restore FDH-2 activity to parental level. This complementation analysis also indicates

that the plasmid pSE-148 is smaller than the plasmid pSE-151, a small difference not detected by agarose gel electrophoresis.

In order to determine the other end of the hydB gene, presumably the 3'end, the 2.8 kb SAlI fragment from plasmid pSE-125 was used (Fig. 5). Attempts to delete from SalI end had resulted in instability of the plasmid DNA and/or cell lysis of the transformants containing these deleted plasmids. The most probable cause of this result may be the multi-copy nature of the plasmid vector, pBR-322. Since most of the proteins that participate in hydrogen metabolism are membrane-bound, production of a truncated protein, produced from a plasmid, in which the gene is partly deleted, may interfere with membrane architecture. In order to overcome this problem, a low-copy plasmid vector was constructed using plasmid vector pUCD-2 (14) as the starting plasmid. The available low-copy vectors are much larger in size and/or they have limited utility in this study, due to the position and occurrance of restriction sites (51). Plasmid pUCD-2 is 13 kb in size and carries the origins of replication from plasmid colE1 (14), a high copy-number E. coli plasmid and pSA, a low copy-number plasmid, originally isolated from Shigalla (66). This plasmid carries the genes coding for kanamycin, ampicillin, spectinomycin and tetracycline (14, 66). In the presence of pSa replication origin this plasmid vector maintains itself at low-copies in

many gram negative bacteria (61). The genes conferring resistance to kanamycin and spectinomycin and the gene(s) resposible for colE1 replication were deleted from the plasmid pUCD-2 by employing various DNA endonucleases and exonucleases. The resulting plasmid, pSE-4 is 4.45 kb in size and contains only the pSa origin of replication. This plasmid carries genes for ampicillin and tetracycline resistance. The tet gene in this plasmid also has an up-promoter mutation, so that transformants containing this low-copy plasmid can be selected at higher tetracycline concentration (greater than 5-7 ug/ml).

The 2.8 kb SalI fragment was ligated into the SalI site of pSE-4 and the resulting plasmid was named pSE-159 (Fig. 7). The plasmid pSE-159 was linearized at the HpaI site (Fig. 7) and enzyme Bal31 was used to delete the DNA from this end. After ligation, strain SE-38 was transformed with the DNA and both the HYD^+ and HYD^- transformants were selected. Plasmid pSE-162 which was HYD^+, was found to lack about Ø.5 kb (Fig. 7) and complemented the defect in the strain SE-38 (Table 8). Among the exonuclease "Bal31-deleted" plasmids which complemented strain SE-38 for hydrogenase, only plasmid pSE-162 restored the normal FDH-2 activity, while other plasmids pSE-15Ø and pSE-151 failed to

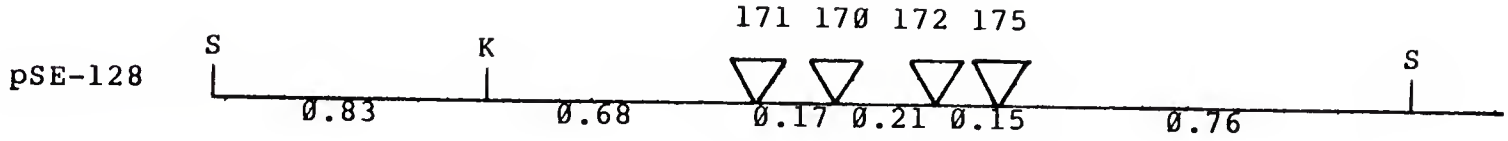

Fig. 8. Map location of transposon Tn5 in hydB gene. Only the 2.8 kb SalI insert DNA containing the hydB gene in the plasmid pSE-128 is shown. The restriction sites are K, KpnI; and S, SalI. The numbers below the line represent the sizes of DNA fragments in kilo basepairs. The numbers above the line are the individual clones with Tn5 inserted in that location.

Table 9. Complementation pattern of mutant strain SE-38 by pSE-128 mutagenized with transposon Tn5

Strain	plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-38	-	66.03	3.02	1.58	26.53
	pSE-128	1,852.83	804.98	96.26	239.88
	pSE-170	72.57	U.D	0.191	13.77
	pSE-171	41.89	U.D	0.168	12.14
	pSE-172	85.53	U.D	0.204	17.79
	pSE-173	59.63	U.D	0.496	41.59
SE-65	-	47.05	8.84	4.40	41.59
	pSE-170	1,120.96	708.14	106.99	83.82
	pSE-172	1,896.27	731.97	107.85	119.39

a, nanomoles of tritium exchanged per min per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.
U.D, undetectable

do so. The FDH-2 activity in these strains was twice the amount present in the mutant strain alone.

In order to further establish that the hydB gene is located at about Ø.7 kb from the KpnI site (Fig. 7), plasmid pSE-128 was mutagenized with transposon Tn5. The transposon Tn5-mutagenized plasmid was used to transform strain SE-38 and the plasmids carrying Tn5 in the hydB gene were selected (Table 9). All four plasmids failed to complement the hydB mutation in strain SE-38 for hydrogenase and other H_2-dependent activities. The plasmid DNA from these clones were analyzed to determine the position of transposon Tn5 (Fig. 8). Transposon Tn5 was located within the segment of the DNA coding for hydB gene, as evidenced from enzyme Bal31 deletion studies (Fig. 7). These results suggest that one end of the hydB gene lies at about Ø.68 kb from the KpnI site (Fig. 8). The other end of the hydB gene ends at about Ø.5 kb from the SalI site (Fig. 7, Table 8).

Identification of Gene-Product Produced by the hydB Gene.

Using maxi-cell technique, it is possible to identify the proteins produced by the genes encoded in a plasmid (54). This technique is generally used to identify the gene-protein relationship. For aerobic and anaerobic expression of the genes in the plasmid, strain CSR-6Ø3 carrying plasmids were grown as described in the Methods section. Plasmid vector pBR-322 produced two proteins as

identified by maxicell technique. The bla gene-product is a 28 kd (kilo dalton) ampicillinase protein and a 38 kd protein is produced by the tet gene. In some experiments, a 31 kd protein was also detected among the proteins produced by plasmid pBR-322, which is the precursor protein for ampicillinase. These proteins are expresssed both anaerobically and aerobically (Figs 9, 1Ø).

Plasmid pSE-125 produced two proteins; namely a 28 kd ampicillinase protein and a 32 kd protein (Fig 9). The tet gene product was absent because the chromosomal DNA containing hydB gene (Fig. 4) was cloned into the SalI site present in the tet gene. The proteins produced from various subclones are presented in Fig. 9. The cells of strain CSR-6Ø3, that carried these subclones were grown anaerobically. Another protein with a molecular weight of 44 kd was detected in the extracts from the plasmid pSE-125 containing cells. Plasmid pSE-126 produced both the 32 and 44 kd proteins at higher levels than produced from plasmid pSE-125, apart from the 28 kd ampicillinase protein. The 32 kd protein was not produced by CSR-6Ø3/pSE-127 since this plasmid lacked the 2.8 kb SalI fragment carrying the hydB gene (Fig. 4). Plasmid pSE-129, in which the 2.8 kb SalI fragment was reversed as compared to plasmid pSE-128

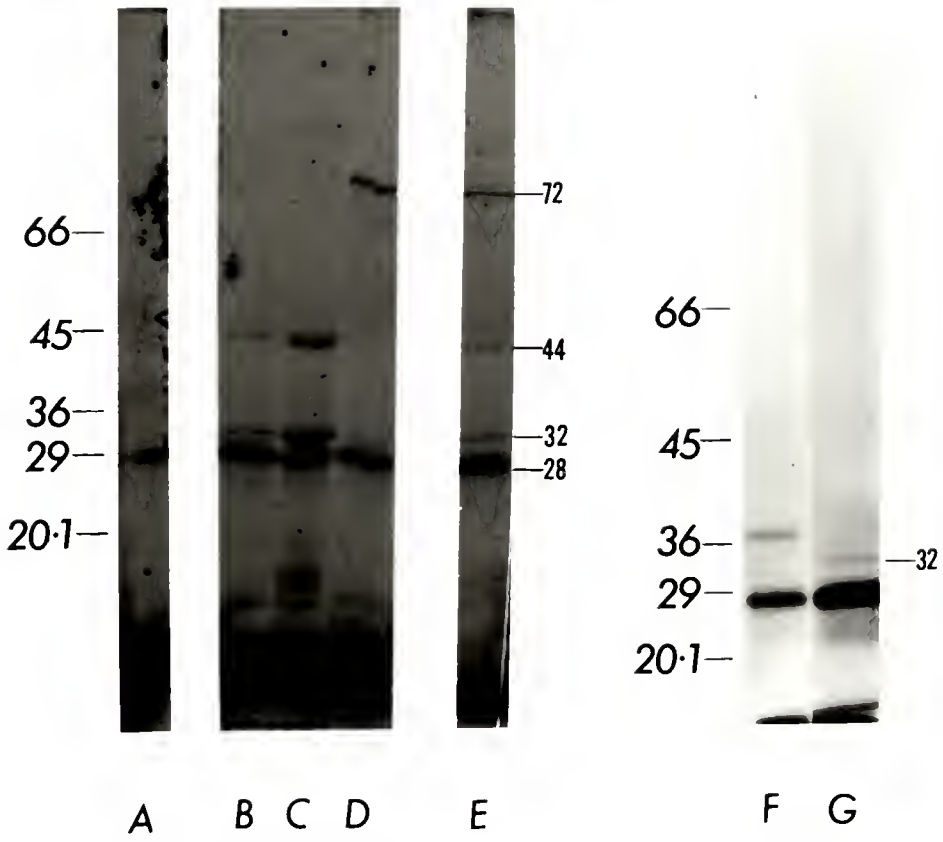

Fig. 9. Protein products produced by plasmid-encoded genes. Plasmids are subclones derived from plasmid pSE-22 (Fig. 4). The cells harboring these plasmids were grown and processed anaerobically (see Methods section for details). Molecular weight of the proteins is in kilo daltons (kd). A, plasmid pBR-322; B, plasmid pSE-125; C, plasmid pSE-126; D, plasmid pSE-127; E, plasmid pSE-129; F, plasmid pBR-322 and G, plasmid pSE-132.

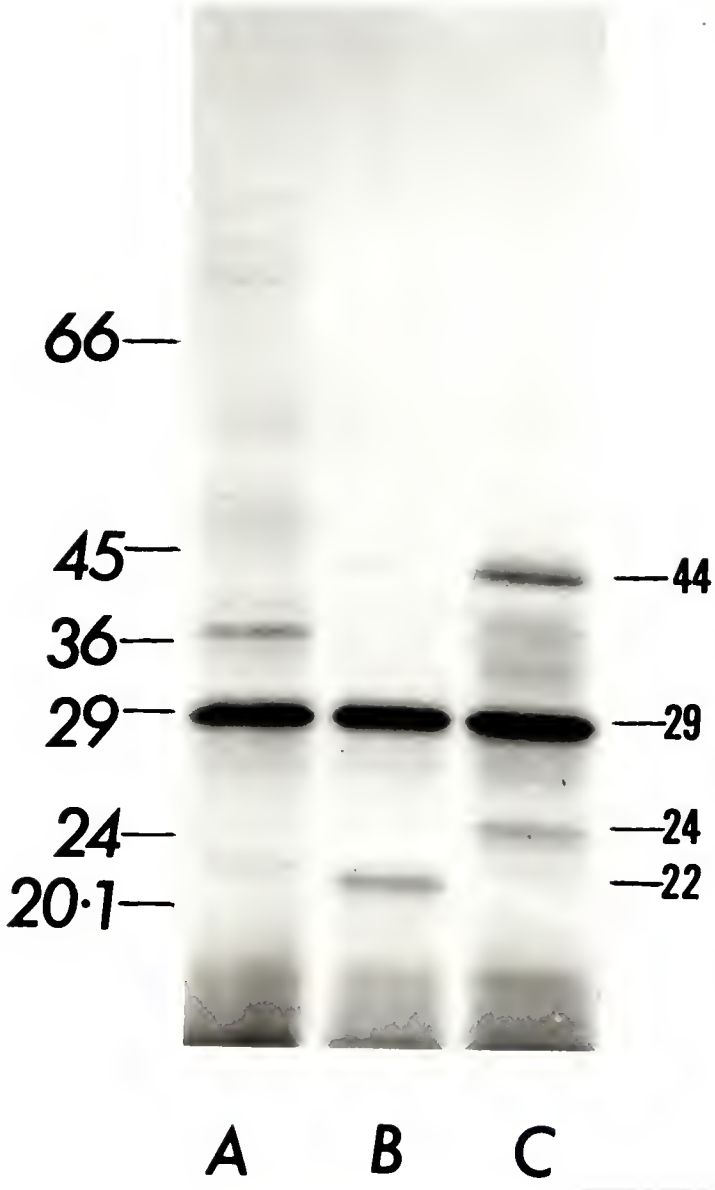

Fig. 1Ø. Protein products produced from plasmid-encoded genes. The cells carrying these plasmids were grown aerobically (see Methods section for details). Molecular weight of proteins is in kd. A, plasmid pBR-322; B, pSE-125 and C, plasmid pSE-126.

(Fig. 4), also produced the 32 kd protein. Plasmid pSE-132 (Fig. 5), which carried only a 1.97 kb insert also produced the 32 kd protein. When the cells harboring plasmid pSE-125 were grown aerobically the 32 kd protein was not produced, suggesting that the hydB gene was expressed only anaerobically (Fig. 10). These results suggest that the 32 kd protein is produced by hydB gene only under anaerobic conditions. The origin of the 44 kd protein produced by plasmids pSE-125 and pSE-126 is not known. The aerobically grown cells of strain CSR-603/pSE-125 (Fig. 10) did not produce the 44 kd protein, but a 22 kd protein appeared. From the extracts of aerobically grown strain CSR-603/pSE-126, 44 and 24 kd proteins were produced.

Identification of a New hyd Gene, hydF.

About 10% of the hydB mutant strains complemented by plasmid pSE-125 (Fig. 4) were not complemented by plasmid pSE-132 (Fig. 5, Table 10) for hydrogenase and hydrogen dependent activities (data not shown). Strains SE-65 and SE-67 were studied as representative of this new class of mutants (Table 1). To further characterize the mutant strains, plasmids pSE-131 (Fig. 7) and pSE-137 were used to transform into these strains. Plasmid pSE-137 was constructed by ligating the 0.83 kb SalI-KpnI fragment (not present in plasmid pSE-132) from plasmid pSE-125 (Fig. 5) into appropriately digested plasmid pUC-19. Biochemical

Table 10. Biochemical characterization of hydF mutant strains SE-65 and SE-67 and complementation analysis

Strain	plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	383.20	94.21	197.40
SE-65	-	47.05	8.84	4.40	32.17
	pSE-125	2,107.16	660.30	51.28	248.58
	pSE-131	101.51	U.D	0.94	29.03
	pSE-132	60.88	U.D	0.46	15.57
	pSE-137	25.40	U.D	1.90	31.45
SE-67	-	20.00	U.D	1.18	12.14
	pSE-125	1,862.69	827.36	48.97	160.30
	pSE-131	30.57	U.D	0.20	29.03
	pSE-132	52.61	U.D	0.07	16.45
	pSE-137	55.67	U.D	3.38	12.68

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.
U.D, undetectable

properties of two representative mutant strains with and without these plasmids are presented in Table 1Ø. The only plasmid which complemented the hyd defect in strains SE-65 and SE-67 was the plasmid pSE-125. These results identify the new gene and suggest that recognition site for KpnI lies within the gene. Since other hyd genes, hydC, hydD (68) and hydE (36) were already identified in E. coli, this new gene is named hydF.

In order to further establish the existence of hydF gene, segments from the 2.8 kb SalI fragment from plasmid pSE-125 were deleted using enzyme Bal31. These deletion plasmids exhibited DNA rearrangements and did not complement the hydF mutation in either strain SE-65 or strain SE-67. In order to eliminate the problems associated with plasmid pBR-322 (a high copy-number vector plasmid), the low copy vector plasmid pSE-4, described previously, was used. Plasmid pSE-159 (Fig. 11), which carries the 2.8 kb SalI fragment, was linearized at HpaI site and digested with Bal31 for varying lengths of time. This Bal31-digested DNA was ligated and used to transform the strain SE-38. The HYD^- (as FHL^-) transformants were selected. Plasmid DNA was isolated from individual $hydB^-$ clones and transformed into strain SE-65. The transformants were analyzed for both hydrogenase and FHL activities. The smallest plasmid DNA which complemented the hydF mutation in strain SE-65 was

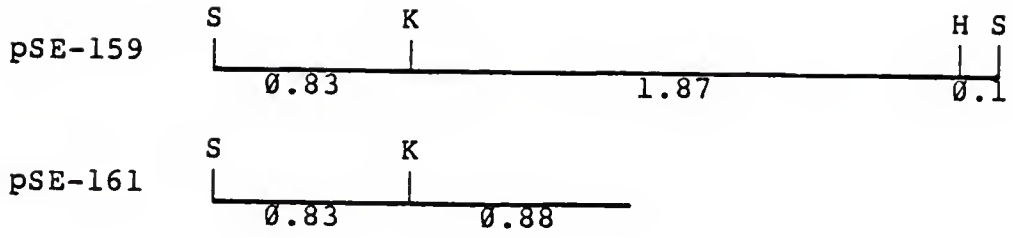

Fig. 11. Chromosomal DNA insert present in a deletion plasmid derived from plasmid pSE-159 by exonuclease Bal31 digestion. Plasmid pSE-159 was linearized at the HpaI site before digestion with enzyme Bal31. The vector is plasmid pSE-4 and is not shown. The restriction sites are H, HpaI; K, KpnI and S, SalI. The numbers below the line represent the sizes of DNA fragments in kilo basepairs.

Table 11. Complementation of hydF mutant strain SE-65 by recombinant plasmids constructed by "Ba131-deletion"

Strain	plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-65	-	47.05	8.84	4.40	32.17
	pSE-125	2,107.16	660.30	51.28	248.58
	pSE-161	1,922.63	624.12	119.34	119.69
SE-38	pSE-161	77.58	U.D	0.01	14.21

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.
U.D, undetectable

isolated and mapped with restriction endonucleases. Plasmid pSE-161 carried an 1.64 kb DNA insert and had lost about 1.16 kb from the 2.8 kb SalI insert DNA present in the starting plasmid pSE-159 (Fig. 11). Plasmid pSE-161 complemented the hydF mutation in strain SE-65 for hydrogenase, HUP and FHL activities, while it failed to do so in the hydB mutant strain, SE-38 (Table 11). These results clearly show that the gene, hydF, is essential for the production of hydrogenase in the cell and is adjacent to the hydB gene.

In order to further demonstrate that hydF gene is different from the hydB gene, because of the proximal location of the two genes, plasmid pSE-128 was mutagenized with transposon Tn5. The Tn5 mutagenized plasmid was used to transform strain SE-65 and kan^R and Ap^R transformants were selected. They were replicated onto HF medium and individual HUP^- colonies were selected. Plasmids, defective in hydF gene due to Tn5 insertion were isolated from these clones and the location of Tn5 in the plasmids were mapped with restriction endonucleases. Three of these plasmids were chosen for biochemical characterization. In plasmids pSE-175 and pSE-176, the transposon Tn5 was mapped at about Ø.17 kb and Ø.42 kb, respectively, from the left SalI site as presented in Fig. 12. In plasmid pSE-177 the transposon was located at about Ø.51 kb to the right of the KpnI site as presented in Fig. 12. Biochemical properties of strain SE-65

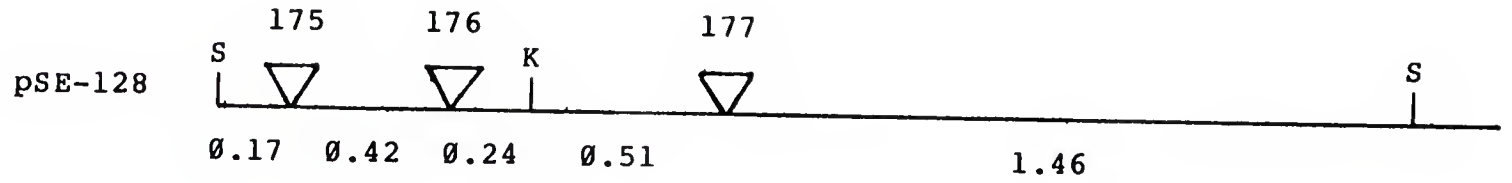

Fig. 12. Map location of transposon Tn5 in the hydF gene. Only the 2.8 kb SalI insert DNA in plasmid pSE-128 is shown. The restriction enzyme sites are K, KpnI; and S,SalI. The numbers below the line represent the sizes of DNA fragments in kilo basepairs. The numbers above the line are the individual clones with Tn5 inserted in that location.

Table 12. Complementation of hydF mutant strain SE-65 by plasmid pSE-128 mutagenized by transposon Tn5

Strain	plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	993.82	159.71	197.40
SE-65	-	47.05	8.84	4.40	32.17
	pSE-128	1,663.28	684.20	88.24	286.39
	pSE-175	928.33	140.10	25.21	17.51
	pSE-176	395.78	58.22	10.21	9.70
	pSE-177	121.36	U.D	5.47	16.35
SE-38	-	66.03	3.02	1.58	26.53
	pSE-128	1,852.38	804.98	96.26	239.88
	pSE-175	1,706.29	1,021.42	80.79	45.40
	pSE-176	1,604.20	1,059.31	83.07	36.24
	pSE-177	2,085.81	800.12	80.42	15.89

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H2 produced per min per mg protein.
U.D, undetectable

carrying these plasmids are presented in the Table 12. While plasmid pSE-177 failed to complement the hydF mutation in strain SE-65, this strain with the other plasmids, pSE-176 and pSE-175 showed partial hydrogenase activity. The HUP and FHL activities of these strains were proportional to the levels of hydrogenase activity present in the cell. The plasmid pSE-176 complemented the hydF mutation in strain SE-65 for hydrogenase up to 24% of the activity as observed with the same strain carrying the plasmid pSE-128 and the HUP activity increased to 58 units from undetectable levels (Table 12). Similarly, complementation by the plasmid pSE-175 gave rise to 59% of hydrogenase and 20% of HUP activities as compared with the strain SE-65 carrying the plasmid pSE-128 (Table 12). The transposon insertion in the hydF gene had also abolished FDH-2 activity in both strains SE-65 and SE-67. Plasmids pSE-175, pSE-176 and pSE-177 complemented the hydB mutation in strain SE-38 for hydrogenase and FHL activities. The FDH-2 activity was not restored by these plasmids in strain SE-38 (Table 12).

The protein product produced by hydF gene was not identifiable by maxi-cell technique. Plasmid pSE-125 (Fig. 4) contains the hydF gene (Table 10) but not plasmid pSE-132 (Fig. 5, Table 10). The only proteins identified in strain CSR-603 containing plasmids pSE-125 and pSE-132 were the 28 Kd ampicillinase protein and the 32 kd hydB gene product (Fig. 9). No other protein band which was present

with plasmid pSE-125 was missing in CSR-6Ø3/pSE-132. The hydF gene-product may be produced in few copies and has escaped the detection by maxi-cell technique.

Characterization of fhlA Gene.

Among the hydB mutant strains, one strain, SE-68 (hydB1Ø8 fhlA1Ø1, Table. 2) was found to be complemented for hydrogenase activity, but not for FHL activity by plasmids pSE-125 and pSE-13Ø (Fig. 4, Table 13). Plasmids pSE-128 (Fig. 4) and pSE-133 (Fig. 13), which carry a 3.7 kb ClaI DNA fragment from plasmid pSE-128 in the ClaI site of the vector plasmid pBR-322, were able to complement the mutation in the strain SE-68 for both hydrogenase and FHL activities (Table 13). Although plasmid pSE-13Ø (Fig. 4) had an extension of Ø.97 kb from the SalI end of plasmid pSE-125 (Fig. 4), it failed to complement the FHL defect in strain SE-68. By extending the Ø.97 kb SalI-PstI fragment present in plasmid pSE-13Ø to about Ø.38 kb (PstI-ClaI segment present in plasmid pSE-133), normal FHL activity in strain SE-68 was restored (Table. 13). Plasmid pSE-127, lacking the 2.8 kb SalI fragment (carrying the hydB gene) failed to complement the defects in strain SE-68. These results suggest that strain SE-68 carrries two mutations; one in hydB gene and another in a gene resposible for FHL activity. This gene is named fhlA.

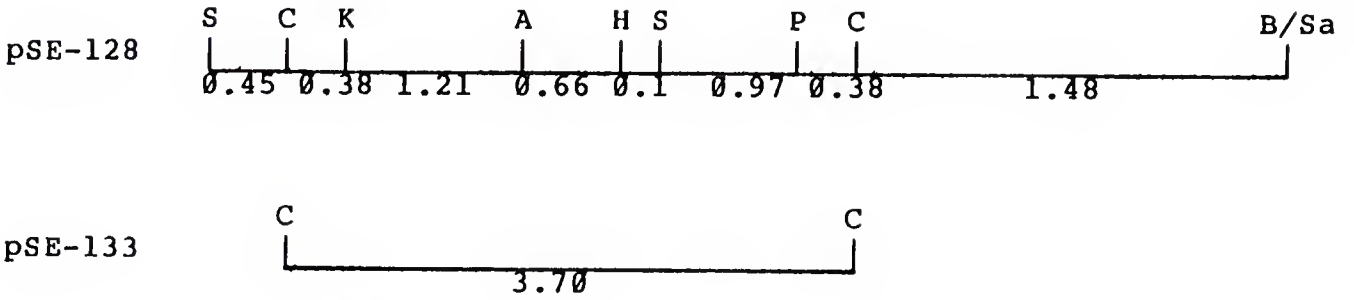

Fig. 13. Chromosomal DNA insert present in plasmid pSE-133. Only the 3.7 kb insert DNA is shown. The restriction sites are A, AvaI; B/Sa, BamHI-Sau3A junction; C, ClaI; H, HpaI; K, KpnI; P, PstI; and S, SalI. The numbers below the line represent the sizes of DNA fragments in kilo basepairs.

Table 13. Biochemical characterization of fhlA mutant strains SE-68 and SE-1174 by complementation analysis

Strain	plasmid	HYD[a]	HUP[b]	FHL[c]	FDH-2[b]
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-68	-	46.49	U.D	0.34	U.D
	pSE-125	2,888.14	1,204.97	6.76	6.0
	pSE-127	<1	U.D	1.29	U.D
	pSE-128	2,513.30	1,034.20	167.36	170.14
	pSE-130	2,659.09	1.191.40	1.90	U.D
	pSE-133	1,454.69	1,415.69	287.40	178.14
SE-1174	-	2,301.84	1,004.64	0.04	U.D
	pSE-125	2,432.22	885.24	U.D	U.D
	pSE-127	2,615.78	836.31	0.18	U.D
	pSE-128	2,384.05	1,156.05	113.11	246.24
	pSE-133	1,904.00	1.040.12	124.96	163.89

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.
U.D, undetectable

In order to further characterize this fhlA gene, it is important to have a mutant strain carrying a mutation only in fhlA gene, unlike strain SE-68 which is a double mutant. A mutant strain, SE-1174 carrying a Transposon Tn10 insertion mutation was constructed by a strategy outlined below. Since the genes hydB and fhlA are adjacent to each other, they should be easily co-transducible by bacteriophage P1. Strain SE-38 (hydB103) was used as the recipient in this transduction experiment with bacteriophage P1. A wild type E. coli strain MC4100 was randomly mutagenized by Transposon Tn10 and tetracycline resistant colonies were selected. Bacteriophage P1 was grown in this pool of Tc^R colonies. Strain SE-38 was transduced for Tc^R and the Tc^R transductants which were also hyd^+ were selected and screened for FHL activity. One such strain SE-1174, a derivative of strain SE-38, was selected as $hydB^+$ and FHL^-. Biochemical properties of this strain, SE-1174 with and without the plasmids pSE-125, pSE-127, pSE-128 and pSE-133, are presented in Table 13. Plasmids pSE-125 and pSE-127 did not complement the defect in strain SE-1174 for FHL activity, while plasmids pSE-128 and pSE-133 were able to complement the defect in FHL activity. Attempts to clone the fhlA gene by itself had resulted in instability and rearrangement of the plasmids. Interestingly, a mutation in the fhlA gene affected the FDH-2 activity and

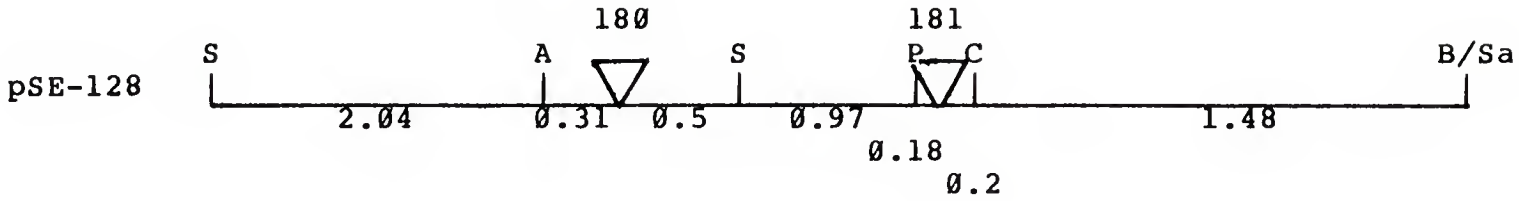

Fig. 14. Map location of transposon Tn5 in the fhlA gene.The chromosomal DNA insert present in plasmid pSE-128 is shown. The restriction enzyme sites are A, AvaI; B/Sa, BamHI-Sau3A junction; C, ClaI; P, PstI; and S,SAlI. The numbers below the line repesent the sizes of DNA fragments in kilo basepairs. The numbers above the line are the individual clones with Tn5 inserted in that location.

Table 14. Complementation of fhlA mutant strains by plasmid pSE-128 mutagenized with transposon Tn5

Strain	plasmid	HYD [a]	HUP [b]	FHL [c]	FDH-2 [b]
Puig 426	-	1,725.24	993.82	159.77	197.40
SE-1174	-	2,301.84	1,004.64	0.04	U.D
	pSE-128	2,384.05	1,156.05	113.11	246.24
	pSE-180	1,881.07	993.99	2.52	U.D
	pSE-181	2,059.49	1,153.31	21.10	U.D
SE-68	-	46.49	U.D	0.34	U.D
	pSE-128	2,513.30	1,034.20	167.36	170.14
	pSE-180	2,783.68	919.29	12.57	U.D
	pSE-181	1,893.34	1,040.08	160.45	212.36

a, nanomoles of tritium exchanged per h per mg protein.
b, nanomoles of BV reduced per min per mg protein.
c, nanomoles of H_2 produced per min per mg protein.
U.D, undetectable

complementation of fhlA mutation by plasmids pSE-128 and pSE-133 (Table 13) had resulted in the restoration of FDH-2 activity.

In order to strengthen the identification of fhlA gene, plasmid pSE-128 was mutagenized by transposon Tn5. The mutagenized plasmid pool was used to transform strain SE-68. Among the Ap^R and Kan^R transformants of strain SE-68, HYD^+, FHL^- clones were screened. From several plasmids which carried Tn5 in fhlA gene, two were selected. The location of Tn5 in these plasmids was mapped with restriction endonucleases (Fig. 14). In plasmid pSE-18Ø, the Tn5 was located with in the 2.8 kb SalI fragment and this plasmid failed to complement the defect in the strains SE-68 and SE-1174 (Table 14). Plasmid pSE-181 which carries the Tn5 close to the ClaI site (Fig. 14) restored FHL activity only in strain SE-68 and not in SE-1174. These results suggest that 2.8 kb SalI fragment contains part of fhlA gene with part of this gene extending beyond the Ø.97 kb SalI-PstI fragment in plasmid pSE-13Ø (fig. 4). Plasmid pSE-133 contains the complete fhlA gene (Fig. 13, Table 13). Failure to restore the normal FHL activity in strain SE-1174 by plasmid pSE-181 may be due to the nature of the transposition by Tn1Ø in the fhlA gene. A truncated fhlA gene product produced from the Tn1Ø inserted chromosomal fhlA gene could lead to the inhibition of the FHL activity and such instances had been demonstrated previously (54).

When analysed by the maxi-cell technique, the plasmid pSE-128 produced 32, 72 and 78 kd proteins apart from the 28 kd ampicillinase protein (Fig. 15). Plasmids pSE-125, pSE-127 and pSE-129 did not produce the 78 kd protein and did not complement the fhlA defect in the strains SE-68 and SE-1174 (Table 13). Plasmid pSE-125 lacks part of the fhlA gene as does plasmid pSE-127 (Fig. 4). In plasmid pSE-129, the fhlA gene is split due to the reversal of the 2.8 kb SalI fragment (Fig. 4). Plasmid pSE-133, which has the complete fhlA gene (Fig. 13) produced a 78 kd protein (not shown). These results show that fhlA gene produces a protein of molecular weight of 78 kd. Plasmid pSE-130 (Fig. 4) produced 66, 44, 38, 34 and 25 kd proteins (Fig. 16). The 38 kd protein is the gene-product of tet gene and the origin of the 34 kd protein is not known. The 66 kd protein was produced only in trace amounts as compared to the 44 and 25 kd proteins, which were produced in larger amounts. The 66 kd protein could be the truncated polypeptide from incomplete fhlA, while the 44 and 25 kd proteins could be the degradative products of the 66 kd protein. The 44 kd protein appeared to be degraded further to a more stable 25 kd protein.

Plasmid pSE-111 produced 32, 78, and 82 kd proteins, apart from the 28 kd bla gene product (Fig. 15). The 72 kd protein produced from plasmid pSE-128 was absent among the products produced from plasmid pSE-111. Plasmid pSE-111

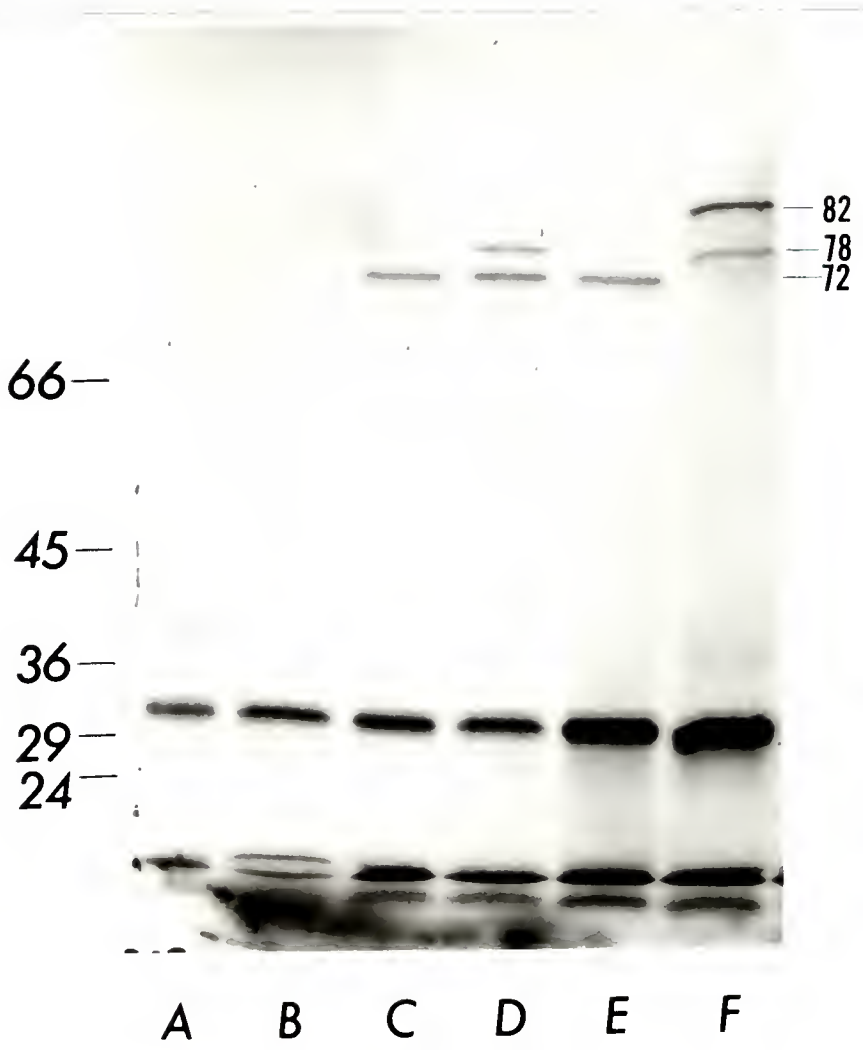

Fig. 15. Protein products produced aerobically by plasmid-encoded genes. Molecular weight of the proteins is in kd. A, plasmid pBR-322; B, plasmid pSE-125; C, plasmid pSE-127, D, plasmid pSE-128, E, plasmid pSE-129 and F, plasmid pSE-111.

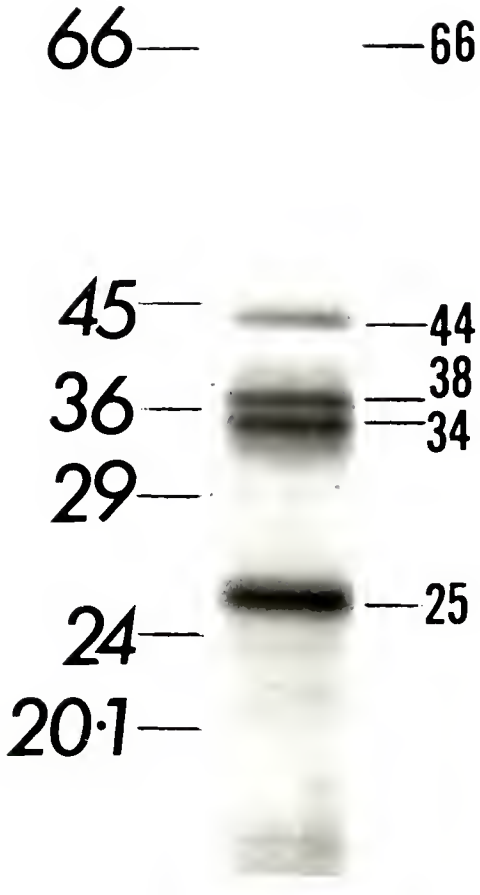

Fig. 16. Protein products produced from plasmid pSE-130. The strain CSR 603/pSE-130 was grown aerobically. The molecular weight of the proteins is in kd.

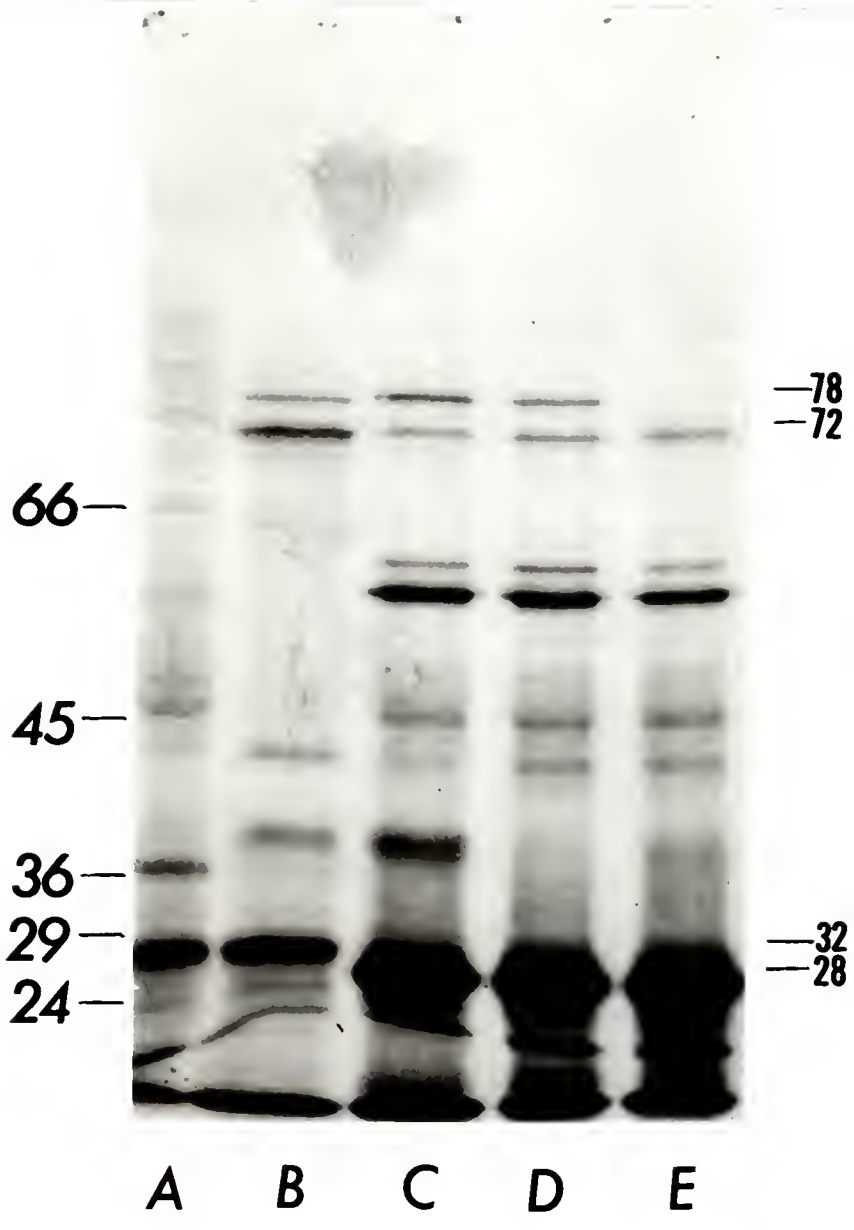

Fig. 17. Protein products produced aerobically by plasmid-encoded genes. Molecular weight of proteins is in kd. A, plasmid pBR-322; B, plasmid pSE-128; C, plasmid pSE-177 (plasmid pSE-128 hydF::Tn5), D, plasmid pSE-170 (plasmid pSE-128 hydB::Tn5) and E, plasmid pSE-180 (plasmid pSE-128 fhlA::Tn5).

carries a 2.1 kb extension from BamHI/Sau3A end of the plasmid pSE-128 (Fig. 2). The 72 kd protein from plasmid pSE-128 or the 82 kd protein from plasmid pSE-111 (Fig. 15) are the products of the same gene fdv (54) and the plasmid pSE-111 carries the complete fdv gene while plasmid pSE-128 does not. The gene-products of both the fhlA and fdv are produced both aerobically and anaerobically (Figs. 9, 15).

Figure 17 shows the protein products produced by plasmid pSE-128 mutagenized with transposon Tn5. Proteins (62, 59 and 46 kd) not produced by plasmid pSE-128 were produced by the transposable element Tn5 (14). The Tn5 insertion in the hydB (plasmid pSE-170; lane D) or in the hydF gene (plasmid pSE-177; lane C) did not affect the expression of fhlA or fdv gene since the products of fhlA and fdv genes (78 and 72 kd, respectively) were produced from the plasmids pSE-170 and pSE-177. An insertion mutation in fhlA gene (plasmid pSE-180; lane E) abolished the expression of this gene as the 78 kd protein was not produced. These results show that both the fhlA and fdv genes are independent operons.

DISCUSSION

Hydrogenase, the enzyme responsible for H_2 metabolism in E. coli, requires the presence of products from several genes which are present at several locations in the E. coli chromosome (59, 66, 77 min of the chromosome). Some of these genes are involved in the production of hydrogenase apoprotein, while others activate the apoprotein to holoenzyme and/or regulate the synthesis of hydrogenase. Since hydrogenase isoenzymes in E. coli are known to contain iron and nickel (6), mutation in the genes which are involved in the transport or processing of these minerals will also affect the H_2 metabolism. It was shown that when E. coli cells were starved for iron, the formate dehydrogenase (FDH-2), hydrogenase and formate hydrogenlyase activites were lost (2Ø) and addition of iron alone to these starving cells allowed the production of all the three enzymes. Since FDH-2, hydrogenase and electron carrier proteins coupling these two enzymes are iron-sulfur proteins (22), the above findings support the concept that iron-deficient cells will not possess these enzyme activites.

Mutations affecting nickel transport into the cells abolished the hydrogenase activities (both FHL and HUP) (68). Two genes are shown to be involved in nickel transport into E. coli cells; hydC gene mapping at 77 min (68), and hydE gene mapping at 59 min (36). The Hyd$^-$ phenotype in these mutant strains of E. coli was reversed by the addition of nickel to the medium.

The two formate dehydrogenases present in E. coli are molybdo-seleno proteins and thus need both molbdate and selenite for activity (15). Formate dehydrogenase-1 (FDH-1) was implicated in the nitrate respiration while formate dehydrogenase-2 (FDH-2) was shown to be part of formate hydrogenlyase system. Some of the chlorate-resistant strains such as chlA and chlE (4, 27), identified as nitrate respiration defective mutants, had low levels of formate dehydrogenase-2 and FHL activities (22). These mutants were found to be defective in processing of molybdate (29). Unpublished results from our laboratory showed that another chlorate-resistant chlD mutant strain of E. coli, defective in molybdate transport (27), produced undetectable levels of FHL activity in the absence of high concentrations of Mo. This phenotype was reversed by the addition of molybdate to the medium. Recently, it was shown that a mutation in an unlinked gene, fhlC, with genes involved in nitrate respiration, abolished both the FDH-2 and FHL-hydrogenase

activities in Mo-deficient medium and the effect was reversed by the addition of molybdate to the culture medium (41). It is imperative that mutations affecting molybdate metabolism will affect the FHL system since formate dehydrogenase-2 is part of that system. It is not known how this regulates FHL-hydrogenase, although it is possible that in the absence of FDH-2, the FHL-hydrogenase is not produced or it is degraded once it is produced.

The pleiotropic regulatory gene, *fnr*, which maps at 29 min of the *E. coli* chromosome, was reported to control the activities of nitrate reductase, fumarate reductase and hydrogenase (39). A mutation in the *fnr* gene reduced the hydrogenase activity to about 15% of the wild type levels (39) and unpublished results from our laboratory, showed that addition of molybdate could reverse the effect of *fnr* gene-product on FHL production.

In this study, three genes are shown to be essential for production of active hydrogenase in *E. coli*. These three genes have been termed as *hydA* (54), *hydB* (54) and *hydF*. Another gene, linked to the *hydB* gene (*fhlA*) is shown to be required for production of FDH-2 and FHL activities. The *hydF*, *hydB* and *fhlA* genes are located at 59 min of the *E. coli* chromosome. The *hydA* and *hydB* genes are not contiguous. The *hydF* and the *hydB* genes comprise adjacent operons. Neither of the three *hyd* genes are found to be involved in the transport of either nickel, a component of hydrogenase,

or molybdenum, a component of formate dehydrogenase-2, since addition of these compounds to the medium did not suppress the effect of the mutation (data not shown). The fhlA gene is linked to hydB gene.

A 2.8 kb SalI-fragment (Fig. 5) from E. coli chromosomal DNA contains the hydB and hydF genes and part of the 5'end of fhlA gene (Figs. 7, 11 and 14; Tables 8, 11 and 14). Based on deletion analyses, it can be inferred that the 5' end of the hydB gene lies at about Ø.76 kb from the single KpnI site (Fig. 7, Table 7) in the plasmid pSE-125, and the 3' end lies at about Ø.5 kb from the SalI site in the plasmid pSE-125 (Fig. 7). The hydB gene codes for a 32,ØØØ dalton protein as observed by the maxi-cell experiment (Fig. 9) and is produced only anaerobically (Fig. 1Ø).

The inability of the plasmid pSE-131 to complement the strain SE-65 and SE-67 (Fig. 7, Table 9) for hydrogenase activity identifies a new gene, hydF, so far unreported. This gene is found to reside in a DNA fragment of 1.3 kb (Fig. 8, 11 and 12). The increase in both hydrogenase and HUP activities with the increasing size of hydF gene (not interrupted by Tn5, Table 12) indicates that the 5' end of the gene is near the KpnI site (Fig. 12) and the gene proceeds through the KpnI site. The 3' end of the gene is obviously closer to the left SalI site as presented in Fig. 12.

A mutation in either hydB or hydF gene not only abolished hydrogenase activity but also reduced the FDH-2 activity (15% of parent values) and complementation of either of the mutations for hydrogenase, restored the FDH-2 activity (Tables 6 and 7). This suggests that a regulatory pathway is coupling the FDH-2 and FHL-hydrogenase, since both are components of FHL system. Interestingly, plasmids carrying wild type hydB and hydF::Tn5 genes (Fig. 12), although they complemented strain SE-38 (hydB1Ø3) for hydrogenase and FHL activites, the FDH-2 activities of the strains carrying these plasmids stayed low (plasmid pSE-177; Table 12). On the contrary, the plasmids wild type hydF gene and hydB::Tn5 (Fig. 8) complemented strain SE-65 (hydF1Ø1) for hydrogenase, FDH-2 and FHL activities (Table 9). It was shown previously that low levels of FDH-2 activity could support normal FHL activity (54). These data suggest a regulatory role for hydF gene-product on FDH-2 production and activity. The hydF gene product is not identifiable by maxi-cell technique, presumably, this protein is produced at a low level in the cell. An expression vector system could be profitably used to detect the gene-product produced from the hydF gene.

A gene responsible for FDH-2 activity and therefore for production of FHL activity in strains SE-68 and SE-1174 was identified as fhlA by complementation analysis (Fig. 13, Table 13). The deficiency in FHL activity may be a

consequence of $Fdh2^-$ phenotype. The plasmid pSE-130 (Fig. 4) which failed to complement the defect in these strains produced only a truncated gene-product of 66 kd (Fig. 16) as compared to the full length 78 kd polypeptide. Extending the partial fhlA gene present in the plasmid pSE-130 by another 0.4 kb led to the production of both the FDH-2 and FHL activites (Table 13). This 0.4 kb DNA segment can easily code for a 12 kd C-terminal part of the protein, in order to make a complete 78 kd protein. These results indicate that the direction of transcription of the fhlA gene is towards SalI, PstI and ClaI sites in the plasmid pSE-128 (Fig. 13). Since the structural gene for FDH-2 was mapped at 92.4 min of the E. coli chromosome (49), this fhlA gene can not be the structural gene for FDH-2 enzyme. The nature of the control the fhlA gene exerts on FDH-2 activity is unknown.

It was shown previously that the plasmid pSE-130 inhibited the FHL activity in the wild-type strain K-10 (54). Now it is clear that the truncated 66 kd protein could lead to the inhibition of FHL activity in strain K-10. Since the FHL system is a membrane complex, comprising FDH-2, hydrogenase and electron carrier proteins, the truncated protein may contribute to the disintegration of the complex resulting in the loss of FHL activity. Another gene, termed as fdv (54), is next to fhlA gene and produces a 82 kd protein. Physiological role of this gene is not known. Clearly, more data are needed to establish the roles of fhlA

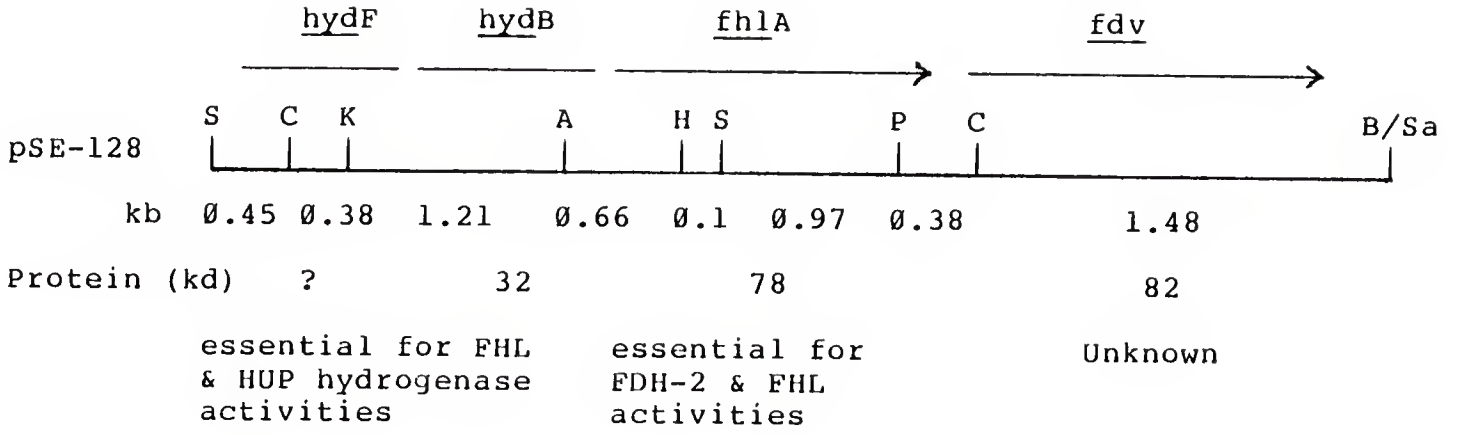

Fig. 18. Summary of the results from this study.

and fdv genes in regulating the FDH-2 activity and the FHL system in E. coli.

Insertional inactivation of hydB and hydF genes by transposon Tn5 did not affect the expression of fhlA or fdv genes present in the plasmid pSE-128 (Fig. 17), suggesting that these genes constitute independent operons. A summary of the results has been presented in the Fig. 18. It is only known that the hydB and hydF gene-products are essential for hydrogenase and all H_2 dependent activities. Their regulatory roles are not understood. Both biochemical and genetic characterizations of the strains carrying lacZ fusions in each of the three genes at chromosomal level, may yield some information on the nature of the control these genes exert on hydrogenase.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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